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Twenty-Four Hour Rhythms in Levels of Putative Inhibitory Transmitters of the CNS in Relationship to the Action of Anti-Convulsive Drugs

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Twenty-four Hour Rhythms in Levels of Putative Inhibitory Transmitters of the CNS in Relationship to the Action of Anti-convulsive Drugs

by

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Dedication

This thesis is dedicated to the memory of my father, Walter A. Piepho, who I am sure would have been proud.

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List of Abbreviations

$\overset{o}{A}$	angstrom unit
AChE	acetylcholinesterase
ACTH	adrenocorticotrophic hormone
ADH	anti-diuretic hormone
AKG	alpha-ketoglutaric acid
AOAA	amino-oxyacetic acid
ATP	adenosine triphosphate
CBLM	cerebellum
ChE	cholinesterase
CNS	central nervous system
COMT	catechol-O-methyltransferase
CQ	corpora quadrigemina
CRF	corticotrophin releasing factor
CS	corpus striatum
CSF	cerebralspinal fluid
CST	central standard time
CTX	cortex (cerebral)
CV	cervical spinal cord
DM	dopamine
DNA	deoxyribonucleic acid
DOPA	dihydroxyphenylalanine
E	epinephrine
ECG	electrocardiogram
ECS	electroconvulsive shock

EDTA	ethylenediaminetetra-acetic acid
EEG	electroencephalogram
et al.	et alia
GABA	4-aminobutyric acid
GABA-T	GABA-transaminase
GAD	glutamic acid decarboxylase
GBL	gammabutyrolactone
GHB	gammahydroxybutyrate
gm	gram
HGH	human growth hormone
HIOMT	hydroxyindole-O-methyltransferase
HVA	homovanillic acid
IPSP	inhibitory post-synaptic potential
LC	lumbar spinal cord
LD50	median lethal dose (50 percent)
LH	lutenizing hormone
LS	lower brainstem
LSD-25	lysergic acid diethylamide
MAO	monoamine oxidase
mcg	microgram
mcM	micromole
MED	medulla oblongata
MES	maximal electroshock
MSO	methionine sulfoximine
N	normal (cycle)

NE	norepinephrine
OHP	hyperbaric oxygen
PCPA	p-chlorophenylalanine
PD50	protective dose (50 percent)
PFFA	plasma free fatty acids
PNMT	phenylethanolanine-N-methyltransferase
R	reverse (cycle)
RNA	ribonucleic acid
SAM	s-adenosylmethionine
TAT	tyrosine aminotransferase
TC	thoracic spinal cord
TCA	trichloracetic acid
TH-HT	thalamus-hypothalamus
US	upper brainstem
WTW	wet tissue weight
5-HIAA	5-hydroxyindolacetic acid
5-HT	serotonin (5-hydroxytryptamine)
5-HTP	5-hydroxytryptophan
5-HTPD	5-hydroxytryptophan decarboxylase
17-OHCS	17-hydroxycorticosteroids

CHAPTER ONE

INTRODUCTION

A. Terminology

1. Types of Biological Rhythms

Halberg (1959) differentiates the terms "rhythm" and "biorhythm" by defining a rhythm as a "statistically validated physiologic change recurring with a reproducible waveform" and by defining "biorhythm" as a rhythm "which persists under a variety of test conditions, such as blinding, constancy (as far as possible) with respect to light (or darkness), and environmental temperature, as well as ad libitum feeding or other conditions and schedules". The frequency or period of a rhythm may be indicated by the adjective preceding it (vide infra).

The word "circadian" is derived from the Latin circa diem, which means "about one day". This term refers to rhythms having a period of 20-28 hours and a frequency of about one cycle per day (Halberg, 1959). Subsequently, this term was used by some workers only in reference to those rhythms which are endogenous and have a characteristic free-running pattern (Aschoff, 1960; Pittendrigh, 1960). The term "diurnal" was then used to indicate rhythms which had only been measured on a 24-hour basis, but it is ambiguous due to its secondary meaning of "daily" as opposed to nocturnal, or nightly. Halberg (1963a) and Mills (1966) consider the use of the term

"diurnal" inappropriate because of this lack of clarity. Wurtman (1967) criticized the ambiguous use of the term "circadian" and suggested that it be restricted only to those studies which qualify as free-running as determined by continuous monitoring. He suggested that the use of the term "daily rhythm" or "24-hour rhythm" be utilized to refer to those systems which are sampled on a single 24-hour cycle.

Halberg has also coined other terms to refer to those rhythms which are non-circadian. The roots of these terms are based on the common prefixes used in physics with reference to light spectra. Ultradian rhythms are those which have a frequency greater than one cycle per day, or a period of between one and 19.9 hours (Halberg, Engeli, Hamburger, and Hillman, 1965). The term "infradian" refers to a rhythm with a frequency of less than one cycle per day, or a period of greater than 28.5 hours (Halberg and Reinberg, 1967).

In addition to these common types of rhythms, Halberg and Reinberg (1967) have subdivided physiological rhythms as follows: "1) a high frequency domain of rhythms with periods shorter than 0.5 hours; 2) a medial frequency domain of rhythms with periods ranging in length from 0.5 hours to 2.5 days, including the regions of ultradian (periods of 0.5 - 19.9 hours), circadian (periods

from 20-28 hours), and infradian (28.5 hours - 2.5 days) rhythms; 3) a low frequency domain of rhythms with periods longer than 2.5 days: inter alia, circasepten (with a period of approximately 7 days), circavigintan (with a period of approximately 20 days), circatrigintan (a period of approximately 30 days), and circannual (a period of approximately one year) rhythms".

2. Other Terminology Used in Reference to Biological Rhythms

The term "cycle" refers to the smallest part of a rhythm which repeats itself. A period is the time interval occupied by a single cycle. The frequency of a rhythm refers to the number of cycles produced in a given time period. Exogenous rhythms are those rhythms which may be altered by alteration of environmental parameters, whereas endogenous rhythms are those which persist under conditions of constant environmental factors, such as constant light or darkness. These latter rhythms are also referred to as "free-running" (Mills, 1966). Phase-shifting is a term used to describe manipulations of the synchronization routine, which may lead to a shifting of the rhythms along the time axis. A sudden shift of twelve hours in a controlled environmental parameter is generally described as inversion. Zeitgeber (sometimes used in the German form as a tribute to Aschoff), or entraining agents

(Pittendrigh, 1960) are terms referring to those environmental factors which are important in setting the phase of a biological rhythm, such as light, temperature, noise cues, feeding schedules, etc.

3. Circadian Versus Local Time

The time axis of any study of rhythms can be expressed by either circadian time or local clock time. Circadian time is based on a time scale utilizing dawn, or first light exposure as time zero. This method of temporal expression is often used by authors in the study of biological rhythms in lower animal forms. Local clock time (eg. Central Standard Time, CST, in this area) will be used throughout this thesis for two reasons: 1) the animals used for these studies were purchased from commercial breeders, and their first exposure to light is therefore unknown, and 2) this type of data is more readily appreciated when expressed on a clock time basis.

B. Historical Aspects

The first known publication in the area of the physiological effects of light was a book written by J. C. Ebermaier, the court-bookseller of Osnabruck, Germany, entitled "Versuch einer Geschichte des Lichtes in Rucksicht seines Einflusses auf die gesamte Natur, und auf

den menschlichen Korper, auber dem Gesicht, besonders."* (Ebermaier, 1799). This book dealt with the effects of light on human subjects as well as with the concepts of circadian and seasonal rhythms in humans. M. Deyeux (1815) published a doctoral thesis dealing with body temperature variations on a 24-hour basis in human subjects. Many studies on body temperature fluctuation followed this initial work of Deyeux's, and by 1925 investigators had examined variability in such phenomena as urinary excretion, excretory rhythms, gonadal weights, and the influence of day-length on migrational habits of birds (Lahr, 1889; Campbell and Webster, 1921, 1922; Simpson, 1924; Rowan, 1925). Cannon's concept of homeostasis was eventually modified to envisage a constantly changing milieu rather than a static condition, and this milieu was believed to have a predominently circadian rhythm, which included some components of shorter or longer duration. In August 1937, the Society for Biological Rhythms held its first meeting at Ronneby, Sweden (Mills, 1966).

The more recent studies in this field will be considered in the Review of Literature.

*"An essay on the history of light, concerned with its influence on man, with or without the sense of sight."

C. Practical Considerations

Environmental alterations will often reflect themselves in the life patterns of the organism. In the case of man, there are variable environmental exposures such as the equivalent periods of light or darkness found at the equator as opposed to the long midsummer and midwinter periods of light or darkness at the poles. Thus, different populations are out of phase with one another (Mills, 1966). These differences presented very little problem to man until he improved travel to speeds which were faster than his capability to adapt to a new environment. When man flies at supersonic speeds, he may cross several time zones, and he subsequently must adapt his internal milieu to a new pattern. In long distance flights, 70% of the travelers experience some physiological discomfort. Strughold (1962) has termed this condition "asynchronosis", and it is exhibited by most higher mammals traversing four or more time zones in succession. He suggests three possible prophylactic measures for this problem: "1) arrive at one's destination several days in advance and allow the biological system to adjust rhythmically to the environment, 2) pre-adaptation or pre-flight synchronization by initiating a sleep/wake schedule identical to that at the point of destination several days

before travel, or 3) the use of mild drugs to accelerate the physiological adjustment". However, "asynchronosis" may also be used by some companies to accelerate the retirement of executives. This is accomplished by extended global travel for the executive, without allowing proper time for adjustment to the local rhythms (Parkinson, 1957).

These environmental problems also affect pilot efficiency. Crane (1963) has described a higher incidence of flight accidents at night than during the day. The increased accident rate coincides with the trough of mental and psychomotor efficiency in man (Browne, 1961). Klein et al. (1970) have noted that pilots exhibit a circadian pattern when tested on a supersonic simulator. The peak and trough of this pattern occur at 2-3 pm and 4-5 am respectively. Eastward, but not westward, flight patterns also caused decreased efficiency due to the increased sleep loss manifested by pilots traveling eastward.

During space travel, man encounters a day of 1.5 hours duration. This has led to work/sleep cycle problems in the Apollo space program. On Apollo 7, cycles were quite irregular and the crew was poorly adapted. The commander fell asleep during his watch, while another crew member was taking d-amphetamine to stay alert during his work period. On Apollo 8, sleep period scheduling was

left at the option of the crew. Secobarbital was included in the medical kit to aid in sedation for the sleep periods. Sleep cycles still varied markedly from the original patterns of the crew, and crew fatigue led to procedural errors and forced "time-changes" in the flight plan. Apollo missions 9 and 10 allowed the crew to sleep simultaneously. This improvement was a factor in the ability of these crews to maintain their original sleep patterns. On the Apollo 11 mission, the crew slept well except for the period of time spent by two of the crewmembers in the lunar module (LM). The environment was too noisy and the space suits were too cold to allow adequate sleep. The LM commander had little, if any, sleep; the LM pilot estimated his sleeping time at two hours (Berry, 1970).

Such studies indicate the need to develop techniques that minimize the undesirable physiological distress associated with man's biological clock, when it is forced to run out of phase with its "normal" environment.

CHAPTER TWO

REVIEW OF LITERATURE

A. Parameters Affecting Biological Rhythms

The synchronization of circadian rhythms has been explained by various mechanisms. One theory (Bunning, 1964) proposed that rhythms are acquired by evolutionary selection, while another (Went, 1960) deals with the possibility of rate-limiting feedback steps, which are self-limiting within a given oscillatory range. A third (Brown, 1960) has been proposed which suggests that circadian rhythms are controlled from the surrounding environment by both strong synchronizers (Zeitgeber), eg. light, and weak synchronizers, eg. barometric pressure (cf. Sollberger, 1969). Some of these parameters and their influence on biological rhythms will be considered.

1. Temperature

The effects of ambient temperature on circadian oscillations appear to be minimal within a 5-10° F. range from the normal environmental temperature. Harker (1958) showed that changes of 5-10° F. in ambient temperature cause no alteration in circadian patterns; however, Brown and Webb (1948) have shown that a large temperature change (to an envirpnmental temperature of 0° C.) can result in delay of the diurnal color changes in Uca pugnax, a species of crab. It appears that temperature changes might become a strong synchronizing factor if they are

sufficiently altered, but small fluctuations in temperature appear to have no effect on biological rhythms (Sollberger, 1969).

A body temperature rhythm is exhibited by many mammalian species, and one might question whether this rhythm is a cause or an effect of the various circadian oscillations.

Body temperature results from the balance between heat production and heat loss. Heat production varies in a circadian fashion and is directly proportional to the body temperature rhythm (Bornstein and Volker, 1926). Kleitman and Ramsaroop (1948) noted similarities in the rhythm of body temperature and heart rate. They administered thyroid hormone over a two week period, and noted a definite association between the pulse rate and the body temperature, Kleitman (1949) demonstrated that the body temperature rhythm of the rabbit is associated with the feeding pattern. The rhythm in mouse rectal temperature has been associated with the motor activity rhythm (Halberg, 1959). This investigation has shown that artificial alterations of body temperature have no effect on the activity rhythm. Brahmachary (1967) suggested that the motor activity rhythm is a primary rhythm, and that the body temperature follows the metabolic activity associated with this primary rhythm.

It is interesting to digress and consider the Feldberg hypothesis concerning temperature regulation. Feldberg and Myers (1964) noted that application of 5-HT close to the hypothalamus increases body temperature, whereas that of norepinephrine (NE) decreases body temperature. They suggested that the biogenic amines might be directly concerned with the control of body temperature.

To summarize, the body temperature rhythm is an effect rather than a cause of the various circadian oscillations; it is also possible that alterations of biogenic amine levels in the vicinity of the "temperature control center" of the hypothalamus are responsible for this rhythm.

2. Illumination

The influence of illumination on biological rhythms should be considered with regard to photoperiod, intensity, and spectrum of the light source.

The importance of photoperiod as a primary Zeitgeber is discussed extensively by Aschoff (1960). The connection between the illumination period and the many light-related circadian variables has been developed in recent years. The effects of light are believed to be mediated via the pineal gland in mammals (Wurtman et al., 1968a). Continual light or increased timing of

photoperiods results in decreased pineal weight, decreased size of the pineal parenchymal cells, decreased pineal lipids, and a loss of the gonadal inhibitory effects of the pineal (cf. Reiter and Fraschini, 1969). The influence of the pineal gland on body growth, pituitary, adrenal, thyroidal, and gonadal function has been pointed out by these investigators. The pathway for the light-mediated effects on the pineal appears to be mediated via the retina, inferior accessory optic tract, and post-ganglionic sympathetic fibers from the superior cervical ganglion to the pineal gland (Kappers, 1960; Moore et al., 1968). Taylor and Wilson (1970) recently demonstrated the inhibitory actions of light on the electrophysiologically-recorded impulses from the pineal gland. They have also confirmed the pathway proposed by Moore et al. (1968) by the use of photic alteration and ganglion-blocking drugs. Light entering the eyes is the important factor in this pathway, although light, which directly penetrates the skull, cannot be discounted (Lisk and Kannwischer, 1964).

The effects of light intensity on circadian periodicity has not been examined as extensively as those of the photoperiod. The intensity of light in most artificially-lit animal rooms ranges from 10-150 foot-candles. Reiter (1969) demonstrated that these levels of light are

inhibitory to the pineal gland.

Marshall and Bowden (1936) described the effects of various wavelengths of light on the timing of the ferret sexual cycle. They found that estrus began earlier in the season in animals exposed to ultraviolet light (3650 Å.), while heat rays and infrared illumination have no effect on this cycle. A similar study by Luce-Clausen and Brown (1939) indicated delayed onset of estrus in rats bred in darkness; normal onset of estrus occurred in rats exposed to visible or infrared illumination. Ott (1964) described some effects of spectral alterations on cells in tissue culture, and on reproductive characteristics and sexual ratio of offspring in both fish and mice. Spalding et al. (1969) described the spectral effects of light on motor activity in albino mice. They noted that the lowest activity occurred in mice exposed to blue or green light, or daylight; intermediate activity occurred under yellow, and the highest activity was noted in either red light or darkness. The retinal receptors of this nocturnal species are mainly composed of rods, which contain a red substance, rhodopsin. This substance absorbs in all of the spectral regions except red. Thus, the red light spectrun is practically equivalent to darkness in this species, (cf. Spalding et al., 1969). Wurtman and Weisel (1969) noted

that rats reared under Vita-Lite (93% of daylight spectrum) have larger gonads and smaller spleens than those reared under a conventional cool-white fluorescent source.

3. Barometric Pressure

Harker (1958) described barometric pressure as a weak synchronizer, suggesting that although it is incapable of controlling a circadian rhythm, it might have an influence on an existing rhythm. Brown (1954) proposed that an increase in the respiratory rates of various species of crabs and salamanders is caused by an increase in the rate of barometric pressure change. Brown et al. (1956a) also noted a direct relationship between the activity of oysters and rate of environmental pressure changes. Hayden and Lindberg (1969) demonstrated that a body temperature circadian rhythm can be entrained in pocket mice (Perognathus longimembria) by fluctuations in ambient barometric pressure in the face of constant ambient temperature and darkness. However, positive entrainment was obtained in only 50% of the mice tested, and the investigators have concluded that the "normal daily cycle of barometric pressure is probably seldom, if ever, an adequate synchronizing agent for circadian rhythms".

4. Sound

Halberg et al. (1954) hold that the sound due to the activity of normal mice has an effect on the activity of blind mice. However, the influence of sound on biological rhythms has rarely been studied. In 1966, "the first clear demonstration that a biological clock can be influenced by sound" was presented by Menaker and Eskin. They used recorded bird songs to entrain activity rhythms in sparrows. It is apparent that sound might be a weak synchronizer.

5. Feeding Habits

Alterations in feeding patterns have been suggested to cause circadian fluctuations. Calhoun (1945) demonstrated that altered motor activity in rodents can occur by changes in feeding schedules. Stein (1951) noted that feeding rhythms in birds will persist in constant light or darkness, even though the sleep pattern has been altered. Harker (1958) proposed that feeding schedules might change biological rhythms associated with motor activity and metabolism in mammals. Honova and co-workers (1968) suggested that the rhythm in the activity of tyrosine transaminase might be controlled by the feeding pattern in both rats and humans. Rohles and Osbaldiston (1969) noted that the feeding patterns in monkeys are independent of light and temperature, but appear to be related to social entrainment. Thus, feeding habits appear to be a weak

synchronizer in biological oscillations. However, more extensive studies involving mammals maintained in the absence of temperature and light fluctuations are not available.

6. Population

The size of the group of animals used in a circadian study might have a weak effect on synchronization of the biological rhythm.

Studies in insects such as the beetle (Park and Sejba, 1935) and the fruitfly (Harker, 1958) indicated that insect activity might be related to the size of the population. Isolation produces aggressive behavior in mice, which is accompanied by changes in serotonin metabolism (Valzelli and Garattini, 1967). These changes in 5-HT metabolism could alter the sleep/wakefulness cycle. The possible role of 5-HT in sleep will be considered elsewhere (vide infra). Harker (1958) noted a reversal of motor activity in those rats, which are low in the social order of their colony. Rohles and Osbaldiston (1969) noted that the individual feeding rhythms of two isolated monkeys coincided when the animals were placed together. They attribute this alteration to the social entrainment of the feeding rhythm, and they intimate that social entrainment might be as important as light or temperature for rhythm entrainment. Quay and co-workers

(1969) examined the effects that population factors of the environment had on the CNS. They found that isolated rats have greater body pineal gland weights, and a higher ratio of pineal to brain weight than do grouped animals. Grouped animals exhibited a greater mean cerebral cortical weight than the isolated animals. No difference was noted in the concentration of pineal 5-HT or in the pineal activity of acetylserotonin methyltransferase (HIOMT) between grouped and isolated rats, but differences in brain acetycholinesterase were noted. The ratio of cortical AChE/ChE decreased in grouped rats; the critical mass for this change appears to be 3-4 rats/cage regardless of cage dimension.

7. Species

The species of an organism appears to have some bearing on the nature of its biological rhythm, eg. man exhibits a diurnal pattern, whereas rodent species exhibit a nocturnal one (Harker, 1958). The ability of various animal species to adapt to altered photoperiods is also reflected by the species involved. Phase-shifting leads to a resynchronization of the biological rhythms in two days in spiders (Aschoff, 1963), while about three weeks are required for this process in mice and rats (Massarelli et al., 1970). However,

resynchronization time is not constant for all rat species (Kleitman, 1949).

8. Sex

Sex hormones can affect circadian oscillations. Slonaker (1912) demonstrated that motor activity in the rat increased during the estrus cycle and was maximal at the peak of estrus. Colvin and co-workers (1968) noted circadian alterations in sleep/wakefulness cycles superimposed on the estrus cycle. By means of chronically implanted electrodes, they monitored females and found that the pre-estrus rat exhibits paradoxical sleep in the afternoon followed by an alert pattern in the evening. The day of vaginal cornification is marked by a high percentage of paradoxical sleep after a night in which there is no paradoxical sleep and the animal is alert. The investigators state that "hormonal changes during the day of proestrus appear to increase alertness that night, compensated by an increase in sleep, especially paradoxical sleep, the following day". It is apparent that monthly rhythms influence circadian ones.

9. Other Influences on Biological Rhythms

Kleitman (1949) described many periodic phenomena related to lunar variations, eg. 1) the severity of scarlet fever is related to the blood pH, which exhibits a

rhythm that correlates with the lunar phase, 2) epileptic attacks are more frequent during the full moon, 3) more male and fewer female deaths occur during the period of the full moon. Brown and co-workers (1956b) reported that male rats in a continuous dim light environment exhibited greater motor activity when the moon was below the horizon.

Brown's group have proposed the presence of an "internal clock", by which biological rhythms are controlled independently from environmental time-signals. Various atmospheric rhythms have been proposed to control this "internal clock". The color change rhythm of the crab, Uca, exhibits a tidal rhythm as does its daily oxygen consumption rate. Oysters from New Haven, Conn. were shipped to Evanston, Ill. for such an experiment. The opening of their valves was timed to the lunar cycle of New Haven for two weeks until they phase-shifted to the Evanston lunar cycle (cf. Harker, 1958). Some correlation has also been noted between cosmic ray cycles and metabolic cycles (Brown et al., 1958). Terrestrial magnetism has also been shown to fluctuate rhythmically with solar and lunar periods. The directional movement of the mud snail Nassarius has been shown to be related to this magnetic-lunar cycle (Brown et al., 1960).

Olfaction has been included because of a report in

the literature concerning the relationship between pineal gland activity and olfactory stimulation. Miline et al. (1963) exposed rats to the pungent odor of isonitrile for six hours, and noted atrophic alterations of the pineal parenchymal cells.

The aforementioned factors might function as underlying rhythms which are responsible for some of the biological variations associated with circadian oscillations.

B. Cyclic Patterns in Mammals

1. Rhythms in Physiological Activity

Circadian rhythms in motor activity have been studied extensively in the mouse (Davis, 1933; Johnson, 1939; Park and Woods, 1940; Saelens et al., 1968) and the rat (Slonaker, 1912; Shirley, 1928; Browman, 1937; Quay, 1970). These investigators established that rodents are nocturnal animals with motor activity peaks during the dark phase of an illumination cycle. Holmquest and co-workers (1966) defined the relationship in rats between motor activity and body weight as well as adrenal function. After forty days of random illumination, activity rhythms were lost with no effect on the steady rise of body weight and no effect on the weights of the endocrine organs. Quay (1970) described the effects of pinealectomy on the motor activity rhythms in the rat. He noted that both normal and

pinealectomized rats will reverse their activity rhythms in response to photoperiod reversal, but there was a difference between the two groups with regard to the characteristics of the phase-shifting response.

Curtis (1937) reported motor activity rhythms for both the sheep and the pig; these mammals exhibit peak activity during the day. Lindsley et al. (1964) initiated a diurnal motor activity rhythm in infant monkeys that had lived in darkness for three years. This rhythm took from 3-5 weeks for stabilization following the initiation of a standard light period. Winget et al. (1969) described the correlation between the diurnal motor activity rhythm of the monkey and its deep body temperature rhythm. These workers view the two rhythms as coupled oscillators ("oscillators which are normally synchronized, but may oscillate independently of each other"), which is the same situation that exists in man between human body temperature and activity (Aschoff et al., 1967). In summary, man, sheep, pig, and monkey are all diurnal species, while the rat and the mouse are among the nocturnal species.

Hoshizaki et al. (1969) demonstrated circadian aspects of electroencephalographic patterns, cardiovascular responses, respiration, and food and water intake in the monkey. They noted that the EEG generally shows a waking pattern during the light phase and a sleeping pattern in

the dark phase. They noted peaks of the ECG and blood pressure curves during the light hours. Respiration appeared to peak during the dark phase. Food and water consumption were greater during the light phase.

Rats exhibit a circadian rhythm with regard to self-induced hypothalamic and septal reinforcing brain stimulation (Terman and Terman, 1970). This study was conducted in constant light, sound, temperature, and humidity. The rhythms were similar to those normally seen for motor activity in rodents. Sofia and Salama (1970) demonstrated a circadian rhythm for shock-induced aggressive behavior in mice. This rhythm coincides with the locomotor activity rhythm in mice, and it appeared to be exogenous in nature because it is lost under continuous illumination.

One can generalize that in a given species, most of the physiological parameters appear to peak concomitantly with the peak of motor activity.

2. Rhythms Involving Hormone Levels of Blood and Urine

Circadian variations in the levels of plasma 17-hydroxycorticosteroids (17-OHCS) have been well documented in the human and many species (cf. Krieger, 1970). This biological fluctuation in 17-OHCS levels mimics the rhythm noted for plasma adrenocorticotrophic hormone (ACTH) levels. Since the rhythm of ACTH levels is noted in the absence of

adrenal tissue, it is assumed that the circadian periodicity of plasma 17-OHCS is secondary to that of the ACTH levels (cf. Krieger, 1970).

In man, the rhythm in 17-OHCS levels peaks in the early morning hours, while in nocturnal animals, eg. rat, the rhythm peaks in the early evening hours. The rhythm can be reversed within eight days in either species by a reversal of the photoperiod (cf. Krieger, 1970). The human infant, who exhibits an inconsistent sleep pattern, does not exhibit a circadian cycle in the levels of 17-OHCS. This cycle is developed between 3 and 13 years of age, and its development lags behind the development of a circadian sleep/wakefulness cycle, which occurs at 5-8 months of age (Franks, 1967). However, the infant monkey exhibits a circadian pattern for 17-OHCS levels in the first week of life (Bowman et al., 1970).

The rhythm in plasma 17-OHCS levels is absent in Cushing's disease (Doe et al., 1960), in which pituitary ACTH production increases. The response of humans to ACTH administration is lowered at night, suggesting that a rhythm might also occur in adrenal sensitivity (Perkoff et al., 1959). However, the main factor in the control of 17-OHCS rhythm might actually be a circadian rhythm in the levels of the peptide which releases ACTH, viz., corticotrophin releasing factor (CRF). The sensitivity of the

neurosecretory cells of the anterior pituitary to the effects of CRF might also account for the circadian rhythm (Clayton et al., 1963). The neurohumoral agents which affect the neurosecretion process have been examined. Krieger et al. (1968) noted that atropine and sodium thiamylal both lead to a loss of the peak in 17-OHCS levels. They proposed that cholinergic mechanisms are involved in the release of ACTH and that these might be responsible for the rise of plasma corticosteroids. Krieger and Rizzo (1969) demonstrated that any alteration of 5-HT levels will block the circadian rise in 17-OHCS levels; however, alteration of central NE content had no effect on this rhythm. It appears that both cholinergic and serotonergic mechanisms might be involved in ACTH release.

Zimmerman and Critchlow (1967) studied the effects of stress on the rise of plasma corticosterone levels at different times of the light/dark cycle. They found that, although a circadian rhythm in plasma 17-OHCS levels was present, the stress-induced increases in steriod levels did not differ significantly between peak and trough times of the rhythm.

Bartter and co-workers (1962) showed that the daily rhythms in the urinary excretion of aldosterone, 17-ketosteroids, and 17-OHCS lag two to three hours behind the plasma rhythm of 17-OHCS levels. They noted correlations

between the ACTH rhythm and the rhythms in sodium, potassium and hydrogen ion excretion, the circulatory levels of white blood cells, and the secretion of glucocorticoids and mineralocorticoids. Boyd and McLeod (1964) noted correlations between the circadian rhythm of plasma 17-OHCS and intraocular pressure, with the latter rhythm lagging about four hours behind the former.

The release of human growth hormone (HGH) has also been shown to vary on a circadian basis. Sassin et al. (1969) related the release of HGH to the period of slow-wave sleep. If the sleep pattern is reversed, the rhythm of HGH release is also reversed.

Groot (1967) demonstrated that the ovaries of prolactin-treated (pseudopregnant) rats contain less ascorbic acid and more progesterone at 1600 hours, than at 1000, 1200, or 1400 hours. Endogenous release of lutenizing hormone (LH) is believed to be the cause of this rhythm.

The rate of release of anti-diuretic hormone (ADH) has also been considered as a circadian variable. Zsoter and Sebok (1955) demonstrated a circadian pattern for serum ADH levels in man, with a peak at night. This alteration in ADH levels helps to explain the decreased urinary output associated with the sleeping phase.

3. Rhythms in the Levels of Electrolytes

Mills (1966) described the circadian fluctuations in the levels of urinary phosphate excretion, which exhibit low values during sleep and rise upon waking. This rhythm appears to be related to the behavioral and activity patterns of the subject. Norn (1929) demonstrated that the circadian patterns of urinary sodium, potassium, and chloride exhibited a trough during the sleeping phase with a peak in the waking phase in man. These rhythms are altered by alteration of environmental parameters, and they appear to be exogenous (Mills, 1966). The effects of glomerular filtration rate on these urinary rhythms are uncertain, according to Mills (1966).

Ammonia and acid excretion in man is high at night and decreases in the morning; these rhythms are unaffected by alteration in food intake. The urinary excretions of urea, uric acid, and creatinine are decreased at night. Calcium and magnesium excretion rises during the early daylight hours, and exhibits a nadir at night. The levels of these ions vary markedly with food intake (cf. Mills, 1966).

Min and co-workers (1966) described renal rhythms in the excretion of various ions; these rhythms were monitored in human subjects who were inactive and fed at

constant intervals. Magnesium and calcium excretions were highest in the early morning hours, while peak excretion of either sodium or potassium occurred in the late morning hours. The rhythm of phosphorous excretion exhibited a peak late in the day with a trough in the early morning hours. Fasting resulted in a loss of the excretory rhythms of potassium and phosphorous. Bahorsky and Bernardis (1967) observed that fed rats exhibited rhythms in the serum levels of sodium and potassium with peaks in the early dark phase; however, they found no rhythm in the serum levels of phosphorous or urea nitrogen. Fasted rats exhibited a significant peak in serum sodium in the early dark phase, but the rhythm in serum potassium was lost. It appears that most of the human circadian rhythms of urinary metabolites are also present in nocturnal animals, although they are 180° out of phase with each other as one might anticipate.

4. Rhythms in Constituents of Carbohydrate, Lipid and Protein Metabolism

Blood glucose levels vary in a circadian fashion in the rat (Pauly and Scheving, 1967; Friedman and Walker, 1969a), mouse (Dziekanowska and Andrzej, 1961), chicken (Twiest and Smith, 1970), and cow (Allcroft, 1933). The nocturnal rodent species exhibit maximal levels of serum glucose during the dark, while the latter two diurnal

species exhibit maximal levels during the light. Bahorsky and Bernardis (1967) noted that the circadian pattern of serum glucose levels is abolished in fasted rats, although a definite pattern was found in fed rats similar to that found by other investigators. Jarrett and Keen (1969) noted that normal individuals exhibited diurnal variations in the glucose tolerance test, but hyperglycemic patients did not.

A circadian pattern has been demonstrated for the liver glycogen levels of the rat and the rabbit (Agren et al., 1931). These rhythms were abolished by adrenalectomy. Sollberger (1964) demonstrated circadian variations in hepatic glycogen levels in several species; the rhythm generally peaks during the light phase in the diurnal, and during the dark in the nocturnal species. He contends that the two primary synchronizers for the glycogen rhythm are food intake and light. Fuller and Diller (1970) found that the hepatic glycogen rhythm, in rats fed ad libitum, peaks at 0500-0800 hours, late in the dark phase. In rats fed a single meal from 0800-1200 hours, hepatic glycogen levels exhibited a prolonged peak from 1200-2000 hours, suggesting the importance of food intake in this rhythm.

Fuller and Diller (1970) also noted that plasma free fatty acid (PFFA) levels exhibit a circadian pattern,

inversely related to glycogen levels. They propose that the control of both the hepatic glycogen and PFFA rhythms might be due to food intake, hormonal factors, or neural control via the autonomic nervous system.

Feigin and co-workers (1968) demonstrated that total whole blood amino acid levels in man exhibit a peak at 1200-2000 hours and a trough at 0400-0800 hours. Tewksbury and Lohrenz (1970) demonstrated that urinary amino acid levels exhibit a similar rhythm to those of whole blood, and they propose that a basic rhythm in amino acid levels is normally present, and that the levels are augmented by food intake.

Forsgren (1935) established the presence of circadian variations in albumin content in rodents and man. Nir and co-workers (1969) have shown that pineal RNA levels as well as protein synthesis are depressed by continuous light exposure, but not by an altered illumination schedule; no change in pineal DNA levels was noted. The RNA and DNA content and the level of protein synthesis of animals in continuous darkness and those on an alternating illumination schedule were similar. It appears from these results that continuous light exposure leads to a decreased cellular metabolism in the pineal gland.

5. Periodicity in Enzyme Systems

The activities of various enzymes have been examined on a circadian basis.

Most of the studies of circadian enzyme rhythms in the CNS have been done on the pineal gland. Hydroxyindole-O-methyltransferase (HIOMT), the enzyme which forms melatonin in the pineal, exhibits a circadian pattern in rats. This pattern of HIOMT activity appears to be inhibited by light, since it is abolished in blinded animals or in the presence of continuous light (Axelrod et al., 1963). This exogenous rhythm is also lost by lesion of the accessory optic tract, but not by lesions in the primary optic tract (Chase et al., 1969). The increased activity of HIOMT in the darkness appears to be related to a cholinergic mechanism since atropine methylbromide inhibited the rise in HIOMT activity normally seen during darkness. Activity was restored by the administration of oxotremorine oxalate, a cholinomimetic (Wartman et al., 1969). A different type of biorhythm was noted in the activity of pineal 5-hydroxytryptophan decarboxylase (5-HTPD). Activity of this enzyme is highest at midday and lowest at midnight in nocturnal mammals (Quay, 1963a), in sharp contrast to the rhythm of HIOMT activity. Snyder et al. (1965a) demonstrated that blinding or sympathetic denervation prevent the increased enzymatic activity of 5-HTPD

normally produced by light. The adenyl cyclase activity of the pineal also appears to be sensitive to alterations in illumination since Weiss (1969) demonstrated that rats exposed to continuous light exhibit a potentiation in the norepinephrine-induced activation of this enzyme. Quay (1963b) showed that continuous light decreased activity of succinate dehydrogenase in the rat pineal, which indicates that illumination can affect the metabolism of the Krebs cycle. McGeer and McGeer (1966) noted that the activity of tyrosine hydroxylase, which limits the synthesis of catecholamines, also exhibits a circadian rhythm in the rat pineal. Tyrosine hydroxylase activity is also maximal during the dark phase in this nocturnal species.

Several hepatic enzymes exhibit circadian patterns. Hepatic tyrosine transaminase (TAT) fluctuates on a circadian basis, which appears to be determined by the pattern of food intake (Fuller, 1970). This rhythm persists in the absence of the pituitary gland, adrenal glands, thyroid glands, or the pancreas. The activity of TAT in the brain does not fluctuate, which suggests that the control mechanisms of hepatic TAT activity are not effective centrally (cf. Fuller, 1970). The presence of 24-hour rhythms in the activities of hepatic steriod hydroxylase (Colas et al., 1969) and tryptophan pyrrolase (Rapoport et al., 1966) have also been demonstrated. Radzialowski and Bousquet (1968)

noted that a circadian pattern exists in the activities of the drug-metabolizing enzymes in the rat liver. Maximal activity was noted at 1400 hours. Phillips and Berry (1970) demonstrated a similar circadian pattern in the activity of mouse lever phosphoenolpyruvate carboxykinase, which catalyzes the synthesis of phosphoenolpyruvate from pyruvate.

Van Pilsum and Halberg (1964) noted a circadian pattern in the activity of transamidase in the mouse kidney; highest activity was noted in the light period.

A circadian rhythm in the activity of cholesterol-7-alphahydroxylase, which is controlled by the adrenocortical system and involved in the biosynthesis of bile acids, has been demonstrated (Gielen et al., 1970).

Circadian variation of enzyme activity might be influenced by fluctuations in the levels of available cofactors. The concentration of brain S-adenosylmethionine (SAM), which plays an important role as a methyl donor in several reactions, has been shown to vary on a 24-hour basis. Peak levels were noted in rat brain during the dark phase, and trough levels during the light phase (Wurtman et al., 1970).

6. Periodicity in the Levels of Putative Transmitter Substances and Their Metabolites

The levels of putative CNS transmitters in various areas of the CNS as well as in the whole brain have been

shown to exhibit 24-hour fluctuations by many investigators.

Wurtman and co-workers (1968b) noted a circadian pattern in the plasma levels of the precursor amino acids of monoamine biosynthesis. In man levels of phenylalanine, tyrosine and tryptophan are generally lowest at 0200 hours and maximal at 1050 hours. Methionine, cysteine, and isoleucine levels also exhibit 24-hour fluctuations, while alanine, glycine, and glutamate levels vary slightly. Coburn et al. (1968) demonstrated that the rhythm in plasma tyrosine levels appears to be regulated by dietary protein intake and by the activity of TAT.

Scheving and co-workers (1968a) demonstrated an ultradian pattern for DM levels of whole rat brain.

Norepinephrine levels in the CNS have been shown to vary on a 24-hour basis in most brain regions. However, Scheving and co-workers (1968a) have found an ultradian pattern for NE levels in the whole brain of the rat. The norepinephrine rhythm of the caudate nucleus of the rat is circadian with maximal levels occurring during the dark phase (Friedman and Walker, 1968; 1969a). Manshardt and Wurtman (1968) examined the circadian rhythms of the NE levels in the hypothalamus, midbrain, thalamus, and striatum of the rat. Peaks occurred during the dark phase except in the striatum and midbrain, in which no rhythm was noted. Reis and Wurtman (1968) demonstrated a

circadian alteration in the NE content of the cat hypothalamus and cervical cord. They did not find 24-hour fluctuations in any other area of the cat brain, but they utilized only two sacrifice points on the 24-hour time base, one at the onset of the light phase, the other at the onset of the dark phase; thus, they might have missed peak values during the middle of either phase. This appears to have been the case, since in a study in the cat brain extended to four points, Reis et al. (1968) noted circadian fluctuations in the NE content of the cat cervical cord, pons, substantia nigra-lateral tegmentum, anterior hypothalamus, tuber cinereum, and pineal gland. Ultradian patterns were described for the superior colliculi and the lateral thalamus. Reis and Gutnick (1970) noted that the NE levels of the cervical and sacral-coccygeal areas of the cat spinal cord varied in a circadian pattern peaking at 1900 hours while those of the thoracic cord exhibited a biphasic pattern. Wurtman et al. (1967) examined the factors controlling the circadian fluctuations of the NE content of the rat pineal gland. Norepinephrine levels of this tissue at the end of the dark period were three times greater than those of the light period. They found that the rhythm is abolished by continuous light, continuous darkness or blinding. Rat adrenal epinephrine varies on a 24-hour basis with maximal levels during the light phase

(Scheving et al., 1968b). Pohorecky et al. (1969) noted that continuous illumination or darkness, as compared to cyclic illumination, leads to a decreased activity of phenylethanolamine-N-methyltransferase (PNMT) in the rat adrenal, and to a decreased epinephrine content. The activity of PNMT in the olfactory bulb and tubercle of the rat brain is increased during darkness, suggesting that epinephrine production increases in these areas during darkness.

Circadian rhythms in brain 5-HT levels have been found in the mouse (Albrecht et al., 1956) and rat (Dixit and Buckley, 1967; Scheving et al., 1968a; Friedman and Walker, 1968, 1969a). Maximal levels occur in the light, minimal levels in the dark, in these nocturnal species. Pineal 5-HT levels also vary in a circadian fashion with the peak in the daytime (Quay, 1963a). Snyder et al. (1965b) discovered that this 5-HT rhythm bears an inverse relationship to the pineal melatonin rhythm and that it is abolished by superior cervical ganglionectomy. However, the pineal 5-HT rhythm in immature rats is unaffected by sympathectomy (Machado et al., 1969). Quay (1968) investigated the 5-HT rhythms in various regions of the rat brain and noted that levels are maximal during the light phase in the frontal cortex, hypothalamus, and lateral lower brainstem. He further noted very little rhythmic

alteration in the 5-HT levels of the cerebral cortex. Reis et al. (1969a) observed that the 5-HT content of the cat upper brainstem and telencephalon varied on a circadian basis. Ultradian rhythms were demonstrated in the substantia nigra-lateral tegmentum and in the anterior hypothalamus. In general, 5-HT levels are inversely related to those of the catecholamines in the CNS, suggesting the possibility of different control mechanisms or that the enzymatic systems involved alternately used one or the other amines (Valzelli and Garattini, 1967).

Acetylcholine levels of the rat brain describe similar circadian patterns whether measured by bioassay (Friedman and Walker, 1969b) or by gas chromatography (Massarelli et al., 1970). This rhythm is marked by a peak during the dark, with a nadir in the light.

Wilson (1965) demonstrated a circadian pattern in the primary excretion of histamine in the rat. Maximal levels were noted in the urine during the dark phase. Friedman and Walker (1968) showed that the histamine content of the rat caudate nucleus varied on a circadian pattern with maximal levels during the dark phase. They (1969a) further noted that serum histamine levels exhibited a circadian pattern with maximal levels prior to the onset of the dark phase.

7. Rhythms Involving the Formed Elements of the Blood

In the first hour of wakefulness, the eosinophil and lymphocyte counts decrease, while that of neutrophils increases (Sharp, 1960; Bartter et al., 1962). These changes might be related to the rhythm of 17-OHCS; however, light and social habits appeared to be primary synchronizers for the rhythm in eosinophil count (cf. Mills, 1966).

C. Circadian Rhythms in Drug Response

Several studies have been conducted which indicate the importance of information regarding circadian effects in drug dosage regimens. Holmgren (1933) noted that the effect of insulin and E on rabbit blood sugar depended on the time of their administration. Mollerstrom (1937) recognized the presence of this insulin rhythm in his treatment of diabetic patients. Some drug firms have begun manufacture of "day" and "night" pills to compensate for the diurnal fluctuations in drug metabolism (Sollberger, 1969).

1. Drugs Affecting the Cholinergic System

Pauly and Scheving (1964) demonstrated in rats that the peak toxicity of tremorine occurred between 2200-0400 hours. A trough was obtained in the mid-afternoon. Sublethal doses of tremorine produced tremors after a shorter latency during the dark phase. Walker (1969) noted in mice

that peak toxicity of acetylcholine, pilocarpine, and oxotremorine was greatest during the dark. He also noted that the circadian toxicity curve for atropine sulphate exhibited a pattern inverse to that of ACh with peak toxicity occurring during the light. Spoor and Jackson (1966) demonstrated that the response to the isolated right atrium of the rat to ACh varied with the time of day. A greater bradycardia was noted at 1100 hours than at 2300 hours. Merritt and Sulkowski (1969) reported that the injection of JB-336, an anticholinergic psychotogenic compound, disrupted the circadian pattern of 5-HT levels in the rat pineal gland.

2. Drugs Affecting the Adrenergic System

Wurtman et al. (1970) demonstrated the presence of a circadian rhythm in S-adenosylmethionine, in rat brain, which was markedly affected by the administration of l-DOPA; they suggested that this phenomenon may be important with regard to the therapeutic effects of this compound in Parkinsonism.

Wahlstrom (1964) noted in the canary that MAO inhibition by pheniprazine, pargyline, or nialamide decreased the duration of the motor activity rhythm for a few days and then increased it. A circadian variation in drug response was demonstrated. Greater effects were obtained when drugs

were administered in the late evening than in the early morning.

Saelens et al. (1968) have noted the effects of pargyline and alpha-methyl-m-tyrosine on motor activity in mice. Pargyline was found to cause an increase in the motor activity of the dark phase, while alpha-methyl-m-tyrosine had an opposite effect during the dark phase. The investigators concluded that "adrenergic systems in the brain have a variable influence on locomotor activity".

Black and co-workers (1969) demonstrated a daily rhythm in the effects of reserpine in the rat. The percent of NE depletion by reserpine was greater at night than during the day. They also noted a non-significant decrease in brain NE half-life at night. The effects of reserpine on the sleep patterns of rabbits have been demonstrated by Tabushi and Himwich (1969). They found that reserpine dosage caused a marked suppression of paradoxical sleep. The pattern following reserpine administration is characterized by an alert pattern for 1.5-2 hours followed by slow-wave sleep.

3. CNS Stimulants and Convulsants

Wahlstrom (1968) demonstrated that the activity of amphetamine sulphate in the canary was greater at night than early in the morning.

Webb and Russell (1966) studied the influence of

24-hour rhythms on the convulsant effects of hexafluorodiethyl ether in the rat. Seizure component times were shorter at 2200 hours (dark phase) than at 1000 hours (light phase). The time interval for the occurrence of tonic convulsions and the onset of post-ictal depression was greater in the dark than in the light phase. A circadian fluctuation was also noted in the percentage of animals exhibiting two clonic seizures following a single exposure to the drug. Fifty percent responded at 1000 hours, while only ten percent responded at 2200 hours. Lutsch and Morris (1967) demonstrated that peak convulsant activity of lidocaine hydrochloride in mice occurred at 2100 hours; minimal activity was noted at 1500 hours.

4. CNS Depressants

Davis (1962) observed that both isolated and grouped mice exhibited a similar trough response in pentobarbital sodium sleeping times, but the grouped mice exhibited a longer sleeping time during the day than did the isolated mice. Pauly and Scheving (1964) noted in the rat that the peak circadian toxicity of pentobarbital sodium occurred at 2200-0400 hours, and the nadir in the mid-afternoon. Friedman and Walker (1969a) showed that the longest sleep onset time and the shortest sleep duration following pentobarbital sodium administration in the rat occurred during

the dark phase of an alternating light/dark cycle. An inverse relationship occurred during the light phase. It was further noted that pentobarbital sodium reversed the circadian pattern in body temperature.

Marte and Halberg (1961) described a peak susceptibility to chlordiazepoxide-induced lethal depression in the mouse at 2400 hours. Haus and Halberg (1959) noted that the crest and trough responses in the mouse to lethal depression from ethanol are at 2000 and 0800 hours, respectively.

Morris and Lutsch (1967) demonstrated a 24-hour pattern in the analgesic potency of a standard dose of morphine in the mouse. They found that peak analgesia occurred at 2100 hours with a trough at 1500 hours. Reis et al. (1969b) correlated the daily rhythm in NE levels in a given brain region of the cat and the susceptibility of that region to the norepinephrine-induced action of morphine.

5. Antihistamines

A circadian pattern in the effects of antihistaminic drugs in humans has been demonstrated by Reinberg and Sidi (1965). They adapted six male subjects to a routine circadian cycle for one week and tested the antihistaminic drug, cyproheptadine. The skin reaction to histamine gave a peak response at 2300 hours, but differences

in its time course of action were noted between 0700 hours and 1900 hours.

6. Antipyretics

Reinberg et al. (1967) showed that the duration of urinary salicylate excretion exhibits circadian fluctuations. They tested the rhythm in six human subjects, previously adapted to a programmed illumination cycle. The time course of salicylate excretion was longest when the drug was given at 1900 hours. Radzialowski and Bousquet (1968) noted in the rat that metabolism of aminopyrine is maximal at 0200 hours and minimal at 1400 hours. Adrenalectomy abolished the rhythm, suggesting the possibility that the rhythm of 17-OHCS might have a function in this rhythmic effect.

7. Antibiotics

Wilson (1965) showed that chloramphenicol influenced the pattern of rat histamine excretion in the urine. Chloramphenicol shifted the peak response in this rhythm from the early dark phase to the center of that phase. Krauer and Dettli (1969) noted a 24-hour rhythm in children under one year old for the rate of excretion of sulfisomidine. The rate is twenty percent lower during the night. Halberg et al. (1955) described a 24-hour periodicity in the resistance of mice to an E. coli endotoxin; this type

of altered systemic resistance to bacterial strains might be a partial cause of the circadian effects of antibacterials.

8. Miscellaneous Rhythms in Drug Response

Ertel et al. (1964) demonstrated a circadian effect in lethality following the administration of methopyrapone, an adrenal corticosteroid inhibitor (SU-4885), in mice. Szeberenyi and co-workers (1969) noted that the plasma half-life of SU-4885 in rats was much longer at 2200 hours than at 1000 hours.

In summary, only a small number of drugs from the myriad of therapeutic compounds now in use have actually been examined for their efficacy over a 24-hour period. Many of those examined exhibited a periodicity in response, suggesting the possibility that dosage should be evaluated with reference to the time of administration as well as to the other factors, which are now generally considered in the administration of therapy.

D. Putative Transmitter Substances and Their Relationship to Cerebral Excitability

1. Dopamine

Blaschko (1957) first proposed that 3,4-dihydroxyphenylethylamine, or dopamine (DM), a biochemical precursor of NE, might have a physiological role of its own.

Dopamine has subsequently been shown to be present in the brain of all mammalian species (cf. Hornykiewicz, 1966) and differentially distributed in the CNS. High levels are found in the corpus striatum, substantia nigra, and globus pallidus, whereas low levels are found in NE-rich areas as the hypothalamus (Hornykiewicz, 1966). The turnover of DM is also very high in the striatum, with a half-life of 2-4 hours depending on the technique used (Iversen and Glowinski, 1966).

The DM-rich corpus striatum might have a role in the control of cortical excitability as Umbach (1959) has demonstrated that stimulation of the cat caudate nucleus decreases seizure activity in various brain areas. This restraining effect was confirmed by Jung and Hassler (1960).

The inhibitory activity of DM has been documented in a number of physiological preparations. McGeer and coworkers (1961) found the inhibitory effect of DM to be sixty times that of GABA on the crayfish stretch receptor neuron. It was also found to be ten times as potent as l-NE or l-E in this regard. Iontophoretic application of DM has been used to verify this inhibitory action in various areas of the cat brain, viz. the lateral geniculate nucleus (Curtis and Davis, 1962), cerebral cortex (Krnjevic and Phillis, 1963a), hippocampus (Bloom et al.,

1965), caudate nucleus (York, 1967), and nucleus cuneatus and gracilis of the dorsal spinal column (Steiner and Meyer, 1966). York (1967) also demonstrated that the inhibitory action of DM was blocked by phenoxybenzamine, an alpha-adrenergic blocking agent.

Relationships between central DM levels and motor activity have also been proposed. Everett and Wiegand (1962) found a correlation between the level of brain DM and the degree of motor hyperactivity in mice. Dominic and Moore (1969) have shown that motor activity and brain DM levels fall progressively as the dose of alpha-methyltyrosine is increased. Dopamine has also been implicated as an important compound in dextroamphetamine-induced hyperactivity (Laverty and Sharman, 1965). The extensive literature on the role of DM in the formation of Parkinsonian tremor has been reviewed by Hornykiewicz (1966). The level of striatal DM appears to be critical for proper function in the extrapyramidal system, and decreased DM levels can be found consistently in the corpus striatum of Parkinsonian patients (Bernheimer et al., 1963). Homovanillic acid, the major metabolite of DM in the striatum, also exhibits decreased levels in Parkinsonism, indicating a disruption of DM synthesis in these patients (Bernheimer and Hornykiewicz, 1965).

The role of DM in the alteration of seizure

phenomena has not been extensively studied, although some reports are available. Engel and co-workers (1968) evaluated the effects of electroshock convulsions on the cerebral metabolism of DM. They found that DM levels of the cerebrum are not significantly altered by electroshock, when they are measured ten minutes after the seizure episode. However, homovanillic acid levels are increased 54% over those of control animals, suggesting that the synthesis and metabolism of DM is increased following the seizure. Hanigan (1971) has noted that DM levels are directly proportional to the seizure latency time; thus, it is possible that this post-seizure increase in DM levels may play a role in the refractoriness following the seizure.

Spencer and Turner (1969), on the other hand, have shown that the effects of pentylenetetrazol-induced convulsions are potentiated by the prior administration of dexamphetamine. An intact DM synthesis is necessary for this potentiation, but altered synthesis of either NE or 5-HT had no effect on the seizures. Thus, DM appears to have a facilitatory action in seizures induced by pentylenetetrazol, although its action in electroconvulsive shock appears to be inhibitory to the seizure due to its action in increasing latency time (Hanigan, 1971).

2. Norepinephrine and Epinephrine

The physiological significance of NE was elucidated by Barger and Dale in 1910 when they noted that it mimicked some of the actions of sympathetic stimulation. Von Euler (1946) first described the presence of "sympathin" in mammalian brain and spinal cord. He also described a high concentration of NE in the CNS compared with that of E. The highest levels of NE are found in the hypothalamus, 1-3 mcg. per gram in man (Bertler, 1961), rat (Glowinski and Iversen, 1966), and cat (Bertler and Rosengren, 1959). The midbrain and pons also contain relatively high levels of NE, while low levels are found in the striatum, cerebral hemispheres, hippocampus, and cerebellum (Carlsson, 1959). Fifty percent of the total CNS content of NE is found in the "aminergic" nerve endings (DeRobertis, 1966).

The importance of E in the mammalian nervous system is questionable. Extremely low levels of E are found in the rat CNS, although conversion of NE to E has been demonstrated via the enzyme, phenylethanolamine-N-methyltransferase, or PNMT (Pohorecky et al., 1968). Barchas and coworkers (1969) have noted the presence of a heat-labile substance that inhibits the activity of adrenal PNMT, which they have isolated from the rat brainstem. They suggest that "the low ratio of epinephrine formation in rat brain may be associated with this substance".

Electrophysiological studies on the central actions of NE have often been contradictory. Some investigators indicate that the predominant action of catecholamines on the cerebral cortex is inhibitory (Krnjevic, 1967; Malcolm et al., 1967). On the other hand, recent studies have shown that NE may be excitatory to cortical cells (Roberts and Straughan, 1968; Johnson et al., 1968). These investigators demonstrated that the pharmacological state of the preparation may be crucial in demonstration of the actions of NE. On encephale isolé preparations, twice as many cells were excited by NE as were inhibited. In preparations anesthetized with nitrous oxide-halothane mixtures, 4½ times as many cells were excited by NE as were depressed. However, under barbiturate anesthesia, which is commonly used, eight times as many cells were depressed by NE as were excited.

Bradley and Wolstencroft (1965) found that 48% of pontine and medullar neurons responded to NE with differing responses: 29% were excited and 19% were inhibited. These investigators (cf. Bradley, 1969) reported that 80% of brainstem neurons responded to NE: 20% were excited and 60% were inhibited in this study. Boakes et al. (1968) have shown that the excitatory responses of the brainstem are stereospecific for NE as they only occur with l-NE, whereas the inhibitory responses were found to occur with

either the l- or d- form.

Norepinephrine has also been shown to be depressant in various brain areas, viz. the medial geniculate nucleus of cats (Tebecis, 1967), the hypothalamus (Bloom et al., 1963), hippocampus, caudate nucleus, and mitral cells of the olfactory bulb (cf. Bradley, 1969).

Studies of intact animals have also led to some conflicting reports. Many investigators view NE as an excitatory factor in behavioral studies, while others refer to it as a behavioral depressant. Systemic administration of NE sometimes leads to an arousal response. Administration of l-DOPA and amphetamine result in behavioral stimulation. In spite of these seemingly excitatory phenomena, the direct actions of NE appear to be inhibitory. A review by Himwich and Alpers (1970) provides a thorough discussion of the role of catecholamines in various behavioral disorders. Dewhurst (1968) has proposed a theory which attempts to explain some of the conflicts that have evolved in catecholamine research. He classifies amines as "type A" (fat-soluble), excitant; "type B", with biphasic action; and "type C" (water soluble), depressant. Salmoiraghi (1966) has proposed two types of adrenergic receptors that are responsible for the dual actions of catecholamines in the CNS.

The effects of these catecholamines on various

types of convulsive behavior have been investigated by several researchers. Swinyard and co-workers noted that the intravenous administration of either NE or E in mice raises the threshold for pentylenetetrazol-induced seizures, while only E increased the threshold for MES-induced seizures (Swinyard et al., 1964).

The role of these amines during the course of the seizure is difficult to assess, but in several studies the levels of amines following a seizure have been examined. Breitner and co-workers (1964) reported that NE levels in the rat brain are decreased after multiple shocks delivered over a short period of time. This fact is not surprising since Kopin and Baldessarini (cf. Glowinski and Baldessarini, 1966) have shown release of labelled NE from brain slices into a perfusing medium after low voltage electrical stimulation. Hinesley and co-workers (1968) examined the effects of both electroshock and pseudoshock (a sham shock procedure) in order to assess the actual role of neurotransmitter substances in convulsive seizures. They found that NE levels are significantly decreased in the cerebral hemispheres of shocked animals, but no alteration was noted in the other brain areas examined. These reduced NE levels may reflect an increased turnover rate during the seizure state as has been noted in rats exposed to electroconvulsive shock twice daily for one week

(Kety et al., 1967), or to seizures induced by hyperbaric oxygen (OHP; Neff and Costa, 1967). Partial confirmation of this supposition can be found in the work of Ladisich and co-workers, who have shown that chronic ECS treatment decreased levels of NE, and simultaneously increased the levels of the acid metabolites of NE in all brain parts examined (Ladisich et al., 1969). They also demonstrated that brain homogenates from shocked animals have a lower capacity for NE uptake.

Most of the pharmacological manipulations involving the catecholamines also involve 5-HT, so several studies will be considered under the heading of "biogenic amines", (cf. Section II-D-4). A few studies have been done utilizing specific inhibition of the rate-limiting step in catecholamine biosynthesis. Blenkarn and co-workers (1969) demonstrated that neither alpha-methyltyrosine or dl-DOPA has any effect on OHP-induced seizures. Musacchio and co-workers (1969) have shown that the activity of tyrosine hydroxylase, the rate-limiting step in catecholamine biosynthesis, was increased by 15% in the whole brain of rats exposed to ECS treatment twice daily for one week; activity was increased by 10% in the cortex and by 24% in the brainstem. The aforementioned studies suggest that convulsions lead to an increased NE synthesis and may alter its uptake as well. This process is accompanied by increased

degradation since turnover is augmented in the post-seizure period (vide supra).

3. Serotonin

Serotonin, or 5-hydroxytryptamine, was initially isolated from blood platelets and the intestinal mucosa by Page and Erspamer in 1948. They also noted its vasoconstrictor properties, and Rapport (1949) identified the compound chemically in 1949. Amin and co-workers (1954) reported the presence of 5-HT in the CNS of the dog. Since that time, 5-HT has been found in several plant species, and in all invertebrate and vertebrate species that have been examined (cf. Erspamer, 1966).

The differential distribution of 5-HT in the CNS has been recognized for several years (Gaddum and Giarman, 1965). The pineal gland contains an exceptionally high concentration of 5-HT, 56.9-72.6 micrograms/grams of tissue in the rat (Owman, 1963). Other brain areas rich in 5-HT include the midbrain, hypothalamus, and the septal area. Low concentrations are found in the cerebral cortex, cerebellum, and the CNS white matter (Bogdanski et al., 1957).

The neurophysiological actions of 5-HT on the CNS have been extensively examined. Marrazzi and Hart (1956) suggested that 5-HT might be an inhibitory substance as they found that it depressed evoked transcallosal responses.

Krnjevic and Phillis (1963a) found that 5-HT depressed cortical activity induced by both glutamate and peripheral stimulation. Roberts and Straughan (1967) demonstrated that 5-HT has both excitatory and inhibitory actions on the cat encephale isolé. Hoffer et al. (1968) found that the application of 5-HT leads to activation of cerebellar Purkinje cells. The actions of 5-HT on the brainstem are also seemingly paradoxical. Bradley and Wolstencroft (1965) found that 5-HT application to single neurons of the pons and medulla led to variable effects: 40% of the neurons tested were excited by 5-HT, while 49% were inhibited. The effects of 5-HT on the lateral geniculate nucleus are depressant when stimulation is induced by the optic tract, but no effect is noted with antidromic stimulation or application of excitant amino acids (Curtis and Davis, 1962; 1963). Serotonin is also depressant to the hypothalamus (Bloom et al., 1963), olfactory bulb (Bloom et al., 1964), hippocampus (Biscoe and Straughan, 1965), and spinal cord (Phillis et al., 1968).

Although most of the studies of 5-HT previously mentioned describe the substance as an inhibitory factor, several studies on 5-HT levels in convulsive states have minimized its importance in the production of the seizure pattern. Bonnycastle and co-workers (1957) reported no change in 5-HT levels following ECS seizure activity, but

an increase after diphenylhydantoin administration. Lysergic acid diethylamide, a potent blocker of the action of 5-HT in the CNS, does not affect seizure susceptibility (Lessin and Parkes, 1959). Garattini and co-workers (1960) reported an increase of 5-HT in the CNS following ECS activity, but they are cautious in interpreting these results due to contradictory data presented by other workers (cf. Garattini and Valzelli, 1965, p. 213). Hinesley and co-workers (1968) have clarified this paradox somewhat in demonstrating that CNS levels of 5-HT are elevated significantly by both ECS and pseudoshock procedures. This increase was not seen in animals that were handled only. Thus, stress and handling contribute to these increases in 5-HT levels which were assumed to be due to the convulsion process.

A diet with excess tryptophan, a precursor of 5-HT in the CNS, lowered the hexafluorodiethyl ether seizure threshold in immature rats (Gallagher, 1969). Brain levels of 5-HT are unchanged one hour after exposure to OHP. Neither p-chlorophenylalanine, which decreases central 5-HT levels, nor 5-HTP, which increases them, has any effect on OHP-induced seizures (Blenkarn et al., 1969). Neff and Costa (1967) reported that the turnover rate of 5-HT in the brain is increased in OHP-treated animals. The exact role of 5-HT in these seizure induction methods

deserves further study.

Para-chloroamphetamine, which appears to increase the level of free 5-HT, has anti-convulsant actions, but these require cautious interpretation due to the amphetamine-like actions of this drug on catecholamine receptors. Schlesinger and co-workers have reported that p-chlorophenylalanine increases the susceptibility to audiogenic seizures in mice (Schlesinger et al., 1969). The administration of 5-HTP has been reported to decrease seizure susceptibility (cf. Maynert, 1969; Prockop et al., 1959). The data above indicates that 5-HT might play an inhibitory role in central convulsive mechanisms, but more study is necessary to confirm this hypothesis.

4. The Role of Biogenic Amines in Seizure Susceptibility

The purpose of this section is to review some of the studies which have dealt with simultaneous alterations in the levels of two or more biogenic amines.

Extensive research has been undertaken to examine the relationship of decreased biogenic amine levels to seizure susceptibility. Toman and Everett (1958) found that drug-induced depletion of biogenic amines shortens the electroshock latency time, and that increased amine levels produced an opposite effect. Reserpine, which lowers levels of both catecholamines and serotonin, also lowers

the threshold to both electroshock and chemoshock seizures (Chen et al., 1954). DeSchaepdryver and co-workers (1962) demonstrated a threefold increase in electroshock threshold in reserpinized rabbits, in which catecholamine levels had been restored by administration of DOPA and an MAO inhibitor. When the catecholamine levels are reduced by the administration of alpha-methyltyrosine, an increase in seizure susceptibility occurs (Rudzik and Mennear, 1966). It appears that decreases in the levels of biogenic amines are directly proportional to decreases in the resistance to electroshock or chemoshock seizure induction.

Similar studies have been performed which demonstrate that increased levels of biogenic amines increase resistance to seizure. Kobinger and co-workers (1958) noted that administration of DOPA with an MAO inhibitor elevated the threshold for pentylenetetrazol-induced seizures. However, DOPA alone had no effect on the seizure threshold in mice pretreated with either iproniazid or reserpine (Prockop et al., 1959). Oliver and co-workers (1970) found that tranylcypromine counteracted reserpine-induced increases in the incidence of OHP seizures and the decrease in average onset times. Increased levels of free catecholamines could be a critical factor in seizure reduction. The anticonvulsant action of amphetamine might be due to an action of this type. Administration of other

blockers of catecholamine reuptake, such as imipramine, amitriptyline, and cocaine, have also been shown to decrease the seizure susceptibility (cf. Maynert, 1969). MAO inhibition by either pargyline or iproniazid increases latency time and survival, and decreases the frequency of OHP-induced convulsions. This effect appears to be dissociated from the metabolism of either 5-HT or NE (Blenkarn et al., 1969). This suggests that DM might be the substance responsible for this action. Pfeifer and Galambos (1967) have implicated DM as the substance involved in the actions of reserpine and of MAO inhibitors on seizure susceptibility.

The inverse relationship between catecholamine levels and seizure susceptibility is well illustrated by the actions of metaraminol on seizure susceptibility. This drug, which displaces NE from its intracellular storage sites, first reduces and then increases seizure susceptibility. Thus, NE levels are increased (in the free state) initially, and then depleted as the free NE is broken down and not replaced (Mennear and Rudzik, 1966).

Although many of the studies on the relationship between catecholamine levels and decreased seizure susceptibility have involved MAO inhibition, Lehmann (1967) has also shown that pyrogallol, an inhibitor of COMT, increases the threshold for audiogenic seizures. Furthermore, this

effect is markedly enhanced by the use of imipramine or an MAO inhibitor.

Six genera of mice were evaluated on the basis of their electroshock threshold and their brain biogenic amine levels (Scudder et al., 1966). Those strains of mice that exhibited the longest latency to minimal electroshock seizures also had the highest central levels of biogenic amines, and vice versa.

Thus, it appears that most of the available literature is in agreement on the relationship between the biogenic amines and seizure susceptibility; the levels of these amines appear to be directly proportional to seizure resistance.

5. Glutamate and 4-aminobutyric Acid (GABA)

The actions of both glutamate and GABA will be considered under the same heading since their physiological actions are interrelated. l-Glutamic acid is the main precursor of GABA; this conversion is catalyzed by the enzyme, glutamic acid decarboxylase (GAD), and it is essentially irreversible (cf. Roberts, 1960). The relationship between these two compounds has been amplified further by Roberts (1968), who has proposed that "the variation of chloride ion content at nerve endings may regulate the GAD activity and thus determine the proportions of glutamic acid and

GABA which might be released on stimulation".

GABA was first identified in 1910 by Ackerman and Kutscher. In 1950, two independent research groups localized large quantities of GABA in the mammalian CNS (Awapara et al., 1950). Kuffler and Eyzaguirre (1955) proposed that GABA might play a role in inhibitory transmission in the CNS; this supposition was partially confirmed when GABA was found to be a major component of an inhibitory brain extract called Factor I (Bazemore, Elliot, and Florey, 1957).

Until recently, GABA was believed to be present only in the mammalian CNS, but Scriver and Whelen (1969) found it in rat kidney. Most of the data on GABA levels pertains to the CNS. Fahn and Cote (1968), in a study of GABA distribution in the monkey brain, found the highest levels in the substantia nigra, globus pallidus, and hypothalamus. Certain areas of the thalamus and cerebral cortex contained lower amounts, while white matter contained the lowest concentrations of GABA. In keeping with the proposed inhibitory action of GABA, cerebellar levels are highest in the axons of cells with proposed inhibitory function, viz., Purkinje, basket, stellate, and Golgi Type II cells (Kuriyama et al., 1966a). On the other hand, glutamic acid, a fairly ubiquitous substance, is the most abundant amino acid in brain tissue (Tallan, 1962). Highest levels are found in the cerebral hemispheres, and

lowest levels are noted in the spinal cord (Shaw and Heine, 1965).

Physiological actions of these amino acids have been investigated and these studies tend to strengthen theories concerning their functional roles (vide supra). Glutamic acid leads to a reversible depolarization of spinal motoneurons (Curtis et al., 1960) and cortical neurons (Krnjevic, 1964) when applied extracellularly. Intracellular application has little effect (Coombs, Eccles, and Fatt, 1955). Eccles (1964) proposed that glutamate acts extracellularly by increasing membrane permeability to sodium and potassium ions. The role of glutamic acid as a putative excitatory transmitter has been discussed (Curtis, 1969). On the other hand, the extracellular administration of GABA leads to hyperpolarization and an increased membrane conductance in cortical neurons (Krnjević and Schwartz, 1967), Deiter's cells (Obata et al., 1967), and spinal motoneurons (Curtis et al., 1968a&b). Krnjević and Schwartz (1967) demonstrated that the equilibrium potential for GABA is close to that evoked by direct cortical stimulation. GABA inhibits cortical activity at low dose levels with rapid onset and a transient time course of action; it is capable of blocking most types of cortical activity (Bradley, 1969). Rogozea and co-workers (1969) proposed that GABA depresses hippocampal neurons, and is responsible for supression of

the orienting reflex. Electrophoretic application of GABA to the retinal ganglion inhibits both spontaneous and light driven activity (Straschill and Perwein, 1969).

Some recent studies have attempted to relate the central levels of these amino acids with the functional state of the animal. Jasper and co-workers (1965) measured levels of these substances following their extraction from the pia-arachnoid space. They found that arousal is associated with both a high rate of glutamate release and a low rate of GABA release; the opposite was noted in animals with an EEG pattern of sleep. Stimulation of either the lateral geniculate body or of the cortex, which lead to a powerful inhibition of cortical discharge, caused an increased efflux of GABA (Mitchell and Srinivasan, 1969). Jasper and Koyama (1969) demonstrated that reticular stimulation increases glutamate levels in the superfusate from cat cerebral cortex. They also noted GABA release during sleep periods, confirming the previous studies of Jasper and co-workers (1965). Obata and Takeda (1969) demonstrated the release of GABA into the perfusate of the fourth ventricle following stimulation of the cat cerebellar cortex.

Intracerebral, intracarotid, or intraventricular injection of glutamate produces convulsions, which can be antagonized by GABA administration (Hayashi, 1952, 1958;

Tani, 1954). The intracarotid and intraventricular administration of GABA as well as its topical application to the cortex, increased the threshold for both electroshock and chemoshock seizures (Guleti and Stanton, 1960; Hayashi, 1958; Rech and Domino, 1960). The balance between glutamate and GABA might actually be the key factor with regard to the effects of this amino acid system (vide supra) on cerebral excitability (Roberts and Kuriyama, 1968; Maynert, 1969).

The effects of many convulsion-producing substances and procedures are associated with a decrease in the central levels of GABA. Relationships have been established between lowered GABA levels and OHP (Wood and Watson, 1963; Wood et al., 1969), pyridoxine deficiency (Tower, 1960; cf. Ann. N.Y. Acad. Sci., 166, 1-364, 1969), hydrazide convulsants such as thiosemicarbazide (Killam and Bain, 1957), picrotoxin (Sytinsky and Thinh, 1964; Saito and Tokunaga, 1967; Galindo, 1969), methionine sulfoximine (DeRobertis et al., 1967), pentylenetetrazol (Whistler et al., 1968), bemegride (Whistler et al., 1968), and allylglycine (Alberici et al., 1969). Reserpine, which decreased seizure resistance (vide supra), lead to decreases in GABA levels (Tada, 1963). Conversely, certain substances that increase the central GABA levels have been shown to possess anti-convulsant actions. Substances

of this type include hydroxylamine (Eidelberg et al., 1960), and aminoxyacetic acid (Kuriyama et al., 1966b). Maynert and Kaji (1962) disputed the action of GABA by noting that animals with increased GABA levels were indistinguishable from controls in response to either electroshock or chemoshock. Diphenylhydantoin, a commonly used anti-convulsant drug, increases GABA levels; however, this drug exerts actions on a number of other metabolic processes (Woodbury, 1969). The increase in GABA levels might be the result, rather than the cause of the action of diphenylhydantoin in the control of seizures.

The current status of GABA can be summed up in the following comment by Eugene Roberts (1968): "much more refined systems must be sought in which to measure accurately the total quantity of GABA released in response to an exactly known amount of stimulation. Until such experiments are performed, GABA must remain only a promising candidate for the role of inhibitory transmitter in vertebrates".

4. Glycine

Glycine, the first amino acid to be isolated, was identified in 1820 by Braconnot. He termed the substance sugar of gelatin, and Berzelius (1848) later termed it "glycine". The substance has recently been proposed as an inhibitory transmitter in the mammalian spinal cord. In 1960, Curtis and Watkins described a non-specific inhibitory

action of glycine on spinal nerves. However, subsequent investigation has indicated that glycine may have a role as an inhibitory transmitter in the lumbosacral spinal cord of the cat (Aprison and Werman, 1965; Davidoff et al., 1967; Graham et al., 1967; Werman et al., 1967; 1968). Evidence for this role is presented in recent reviews by Aprison and co-workers (1969a) and Werman and Aprison (1968).

Glycine, the simplest of amino acids, is found ubiquitously in mammals. Its distribution in the CNS of mammals exhibits a rostral-caudal gradient with the highest levels being noted in the medulla oblongata and the spinal cord (Aprison et al., 1968; Aprison et al., 1969b). In general it was found that "glycine concentration was a) two to five times higher in the medulla oblongata, cervical enlargement, and lumbar enlargement than in four brain areas rostral to the medulla oblongata, b) lower in the mid-thoracic cord than in the cervical and lumbar enlargements, and c) generally invariant in the brain areas other than in the medulla oblongata" (Aprison et al., 1969b). The investigators correlated these high levels of glycine with the amount of musculature supplied by the various spinal areas investigated. They found that a positive correlation existed between the number of interneurons and the glycine concentration (Davidoff et al., 1967; Graham et al., 1967).

Glycine has been shown to hyperpolarize both

cortical (Curtis et al., 1968b) and spinal motoneurons (Curtis et al., 1968a; Werman et al., 1968). The reversal potential of glycine hyperpolarization is similar to that of the actual inhibitory processes of spinal motoneurons (Curtis et al., 1968a; Werman et al., 1968). Glycine is a more potent depressant of spinal motoneurons and interneurons than is GABA, and the reverse is true for cortical neurons. Glycine and GABA are equipotent on the Renshaw cell and cuneate neurons of the spinal cord (Curtis et al., 1968b). Glycine also possess potent inhibitory actions on the cuneate nucleus of the medulla (Galindo et al., 1967).

The inhibitory actions of glycine in the spinal cord are prevented by the iontophoretic application of two potent convulsant agents, strychnine (Curtis, 1963; 1969) and picrotoxin (Davidoff and Aprison, 1969). Wood and coworkers demonstrated that glycine can protect rats from the effects of OHP-induced seizures (Wood et al., 1966). Immature rats fed a diet with excess glycine exhibited no change from controls in response to hexafluorodiethyl ether (Gallagher, 1969). Glycine has been shown to protect rats from convulsions induced by homocysteine; however, homoserine, serine, betaine, and glucose all share this action (Sprince et al., 1969). Although some studies indicate that glycine may play a part in convulsive phenomena, much study is needed for the confirmation of this role.

7. Acetylcholine

The intracarotid, intraventricular, or intracisternal administration of acetylcholine (ACh), as well as local application to exposed brain areas, results in precipitation of grand mal seizures, or EEG symptoms similar to those of grand mal epilepsy (cf. Maynert, 1969). Similar results occur when cholinesterase is inhibited in the CNS, allowing an increased central level of ACh (Feldberg and Sherwood, 1954).

Richter and Crossland (1949) have reported that ACh content in the CNS is decreased during the initial shock stimulus, returns to normal levels, and is then decreased again during the convulsive episode. Acetylcholine levels of the brain are also decreased during chemoshock seizures elicited by pentylenetetrazol or picrotoxin (Stone, 1957). Longo (1966) has shown that atropine has no effect on the convulsant response to pentylenetetrazol. Stone has also demonstrated the convulsive effects of tetraethyl pyrophosphate (TEPP), a potent anticholinesterase; he found that these seizures could be antagonized by the administration of atropine. It appears that the ACh content of the brain is decreased following various types of seizure patterns induced by different convulsant procedures. This decreased ACh content is believed to be associated with an increased release and degradation of this neurotransmitter agent;

such a decrease is not unexpected in light of the increased transmission associated with the convulsive episode (Stone, 1969).

Maynert (1969) has evaluated some of the older studies on the role of ACh in seizure susceptibility, and he has noted that many of these studies were performed in anesthetized animals, which show an altered metabolism of ACh. Sobotka (1969) demonstrated that brain levels of ACh in the mouse are increased following administration of MSO, a convulsant substance with a 60-90 minute latent period prior to convulsion. Thus, it appears that ACh levels might be normal or increased prior to the actual convulsive episode, and they are decreased due to the release of the stored ACh during the convulsion. The role of ACh in convulsive episodes has been thoroughly reviewed by Curtis (1969) and Maynert (1969).

8. Generalizations

In general an increased level of any of these putative inhibitory transmitters leads to a decrease in seizure susceptibility; conversely, seizure susceptibility appears to be increased by the administration of putative excitatory substances.

E. Objectives

It is difficult to ascertain accurately the nature of drug efficacy without adequate information regarding the background upon which a drug acts. Inhibitory systems are involved in convulsive disorders and their amelioration, and a number of chemical substances have been considered as inhibitory transmitter candidates in the CNS. It is the purpose of this study:

1) to acquire background information by determining the levels of putative inhibitory transmitters of the CNS as a function of the time of day using a "normal" light cycle, and to examine the effect of a "reversed" light cycle on some of these amine levels.

2) to determine the role of these circadian variations in transmitter levels on the response to maximal electric shock seizures.

3) to determine the diurnal and nocturnal fluctuations in the response of rodents to selected convulsant drugs and to common anti-convulsants in antagonizing maximal electroshock seizures.

CHAPTER III

MATERIALS AND METHODS

A. Basic Experimental Conditions and Procedures

1. Animals

Male albino rats of Sprague-Dawley strain (Holtzman Breeders, Madison, Wisconsin), weighing 275-315 grams, were used for all rat experiments. They were housed in pairs in stainless steel wire mesh cages, 11x9x9 inches. Pel-E-Cel bedding (Paxton Products, Paxton, N. J.) was used as an absorbent material beneath the mesh bottoms of the cages, so that animals had no direct contact with the bedding.

Male albino mice of Ha/ICR strain (A. R. Schmidt Breeders, Madison, Wisconsin), weighing 32-37 grams, were used. These animals were caged in groups of twenty animals each in stainless steel wire mesh cages, 16x6x9 inches. Softwood sawdust was used for bedding material, but animals had no contact with this material, which can influence drug responsiveness (Vesell, 1968).

2. Environmental Conditions

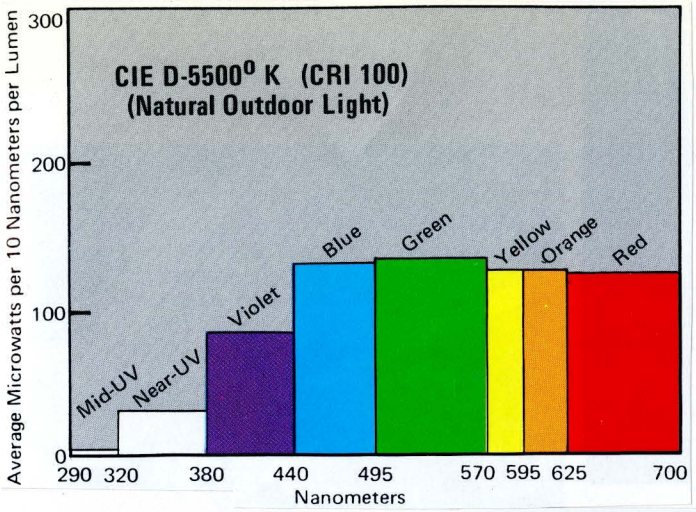
Both mice and rats were given food (Purina Rat Chow) and tap water ad libitum. The composition of Rat Chow consisted of 24% crude protein, not less than 4% fat, 5% crude fiber and 9% ash, with vitamins and minerals added. Feeding, watering, and cage cleaning were performed at various times of the day to prevent these procedures from becoming

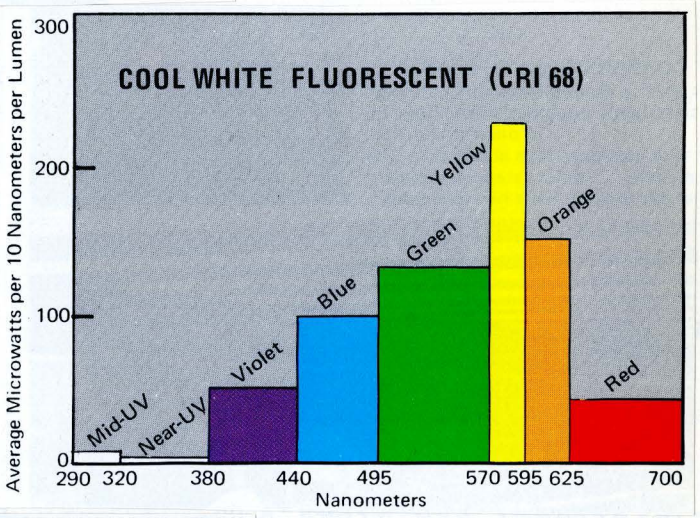
entrainment stimuli. Cage racks were kept in a quiet, well ventilated, windowless room, which measured 9x12x13.5 feet. Temperature was stable at 23 ± 1° C. Ambient humidity was fairly constant at 25 ± 5%, as was the barometric pressure at 732-762 mm. of mercury. These three parameters were recorded on a 24-hour basis by a Lambrecht KG recorder (Gottingen Type 235).

3. Lighting Conditions and Photoperiods

Animals used for the initial studies of dopamine and GABA levels (in corpus striatum, thalamus-hypothalamus, and corpora quadrigemina) were exposed to 85 foot-candles of incandescant light as measured by a Gossen Luna-Pro CdS light meter. Light was provided by General Electric 150 watt light bulbs at a distance of four feet from the cage front. These bulbs had no appreciable effect on the temperature of the room as monitored by the Lambrecht recorder.

Animals for all subsequent studies were adapted under conditions of Vita-Lite (Duro-Test Corp., North Bergen, N. J.) lighting, a fluorescent light source with a spectrum approximating that of daylight. This similarity can be noted in spectra A and C of Figure 1. The lights were placed at a distance of 5-6 feet from the front of the cages, and their output was measured at 100 foot-candles by the Gossen light meter.





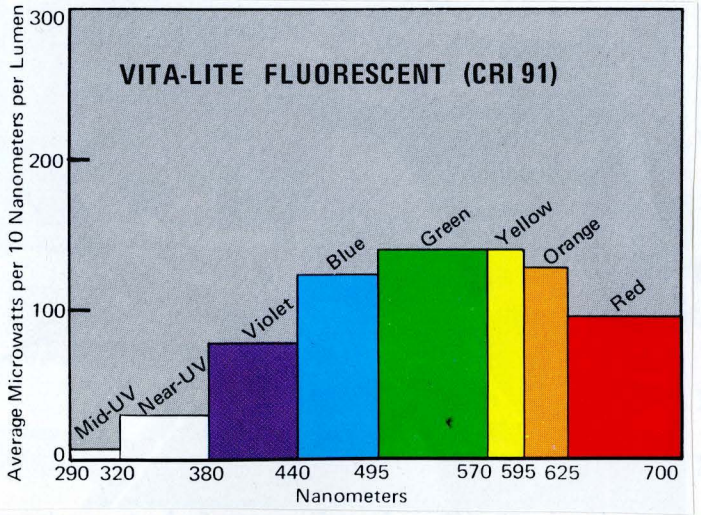


Figure 1. Comparative Spectra of Vita-Lite in relation to other light sources. (Courtesy of Duro-Test Corporation, North Bergen, N. J.)

All animals were adapted for a minimum of three weeks prior to experimentation. Two types of light cycles were used: 1) a normal cycle in which the photoperiod lasted from 0800-2000 hours CST., and 2) a reverse cycle in which the photoperiod lasted from 2000-0800 hours CST. These photoperiods were automatically timed by a Sears Kenmore timer (All purpose 24-hour timer, 15 amps output).

4. Dissection of Tissues

a. Brain Areas

Groups of six rats were killed by decapitation (Harvard Decapitator, Model No. 130 RM) at 3-6 hour intervals around the clock. The skull was opened by an incision with surgical scissors following the midline suture. The dura was incised, and the brain was removed following separation from the cranial nerves. The brain was immediately placed on a cold aluminum block or on a cold glass plate, and rinsed with cold 0.9% saline solution to remove visible blood. It was blotted on filter paper and dissected into the following regions (depending upon the experiment): cerebellum, frontal cortex, corpus striatum, thalamus-hypothalamus, corpora quadrigemina, pons, medulla; upper brainstem consisting of thalamus, hypothalamus, and midbrain; and lower brainstem consisting of pons and medulla (cf. Figure 9 in Zeman and Innes, 1963). Excised tissues

were weighed on a Roller-Smith tissue balance (Model G, 500 mg. counter-weight), and frozen in liquid nitrogen within three minutes after decapitation. Tissues were stored at -25° C. for periods up to three weeks prior to amine quantification.

b. Spinal Cord Dissection

Groups of six animals were killed by decapitation, and the spinal cord was exposed by laminectomy with a fine-pointed surgical scissors. Any excess tissue was trimmed away from the rostral area of the cord. The cord was lifted from the canal by a probe, which had been used to break the dorsal and ventral roots in order that the cord could be romoved en bloc. The cord was rinsed with 0.9% saline solution, and was placed on a cold glass plate. The cord was then divided into the cervical enlargement, lumbar enlargement, and the thoracic spinal cord (cf. Figure 20, Zeman and Innes, 1963). These areas were weighed and frozen in liquid nitrogen within 4-5 minutes following decapitation. They were stored at -25° C. for 1-2 weeks prior to glycine assay.

5. Methods of Amine Analysis

a. Dopamine

Tissues were analyzed for dopamine (DM) by the

method of Sourkes and Murphey (1961). The tissues were homogenized in 5 ml. of 10% trichloracetic acid (TCA) in a glass Potter-Elevjehm homogenizer. The pestle was rinsed with 1.0 ml. of 10% TCA, which was allowed to run off into the homogenizing tube. The tubes were centrifuged at 1000g for 10 minutes in a IEC Model V centrifuge (radius head, 7.5 inch). The supernatant fluid, which contained the catecholamines, was placed in a 50 ml. glass-stoppered centrifuge tube containing 1 ml. of a freshly prepared 0.2% ascorbic acid solution, which prevented the oxidation of DM. The tissue pellet was washed twice with 2 ml. portions of 10% TCA, and the washes added to the centrifuge tube. Standard tubes containing 0, 0.25, 0.50, or 1.00 micrograms of DM were carried simultaneously through the entire method in order to establish the standard curve for DM in this procedure. 2.5 ml. of 10% EDTA solution, one drop of 1% phenolphthalein solution, and 0.5 grams of Woelm alumina (Alupharm Chemicals, New Orleans, La.) were added to each tube. EDTA, disodium form, aided in the chelation of divalent cations, and its presence facilitated consistent recoveries of DM. Woelm non-alkaline alumina was specified because of its low percentage of "fines", which prevent rapid settling, as well as for its neutrality, which allows more rapid pH adjustment. Phenolphthalein was used as an indicator for the following titration. The solutions were titrated to

a pale pink (pH 8.2-8.4) with 5N, and then 1N, sodium hydroxide. This pH range has been shown to be optimal for catecholamine adsorption onto the alumina (Anton and Sayre, 1962). The tubes were then stoppered and shaken at high speed for 15 minutes on an Eberbach shaker. After the alumina had settled, the supernatant fluid was removed by suction with a small-bore glass tube connected to a faucet aspirator. The alumina was washed twice with 5 ml. portions of distilled water, pH 7.0. After removal of the second wash, 5 ml. of 0.5 N acetic acid were added to each tube. The tubes were shaken on high speed for 15 minutes, and were centrifuged 15 minutes at 1000g. This step removed the catecholamines from the alumina and separated the alumina, so that 2.0 ml. of the acid solution containing dopamine could be removed from each vessel sans alumina. Recovery of catecholamines was in the range of 60 $\pm$ 4%.

Two sets of test tubes (18 x 150 mm.) were designated set A and set B. Each tube received 2.0 ml. of 1M sodium acetate buffer, pH 6.0. 1.0 ml. of acid eluate from each centrifuge vessel was added to each tube of sets A and B. Set B tubes were developed for the fluorimetric analysis of DM by the addition of 0.5 ml. of 0.01 N iodine (in absolute ethanol), 0.5 ml. of alkaline sulfite solution exactly three minutes later, and 1.0 ml. of 5N hydrochloric acid,

containing 2 mg./ml. of ascorbic acid, exactly three minutes after the alkali addition. Set A tubes were used as blanks and treated similarly except that the added alkaline sulfite contained 2 mg./ml. of ascorbic acid, and the 5N hydrochloric acid was added just prior to reading the fluorimeter. The tubes were allowed to stand in diffuse daylight, or under an ultra-violet source, for at least forty-five minutes before reading. The fluorophore formed was stable for up to one week. The solution was transferred to a quartz cuvette with a 10 mm. light path. DM fluorescence was read at an excitation wavelength of 330 millimicrons (uncorrected) and an emission wavelength of 375 millimicrons (uncorrected). An Aminco-Bowman Spectrophotofluorimeter (SPF model) was used for all fluorimetric analyses. A number 2 slit scheme was used for DM analysis and the emitted fluorescence was amplified on a RCA 1P21 photomultplier tube.

Treatment of catechols with iodine at weak acid pH leads to formation of products which isomerize in alkaline media to dihydroxyindoles in the case of dopamine-like substances (trihydroxyindoles in the case of epinephrine or norepinephrine). Figure 2 depicts this conversion. In alkali, however, the DM fluorophore will emit light in the same region of the spectrum as will the fluorophores of epinephrine or norepinephrine; therefore, the solution is

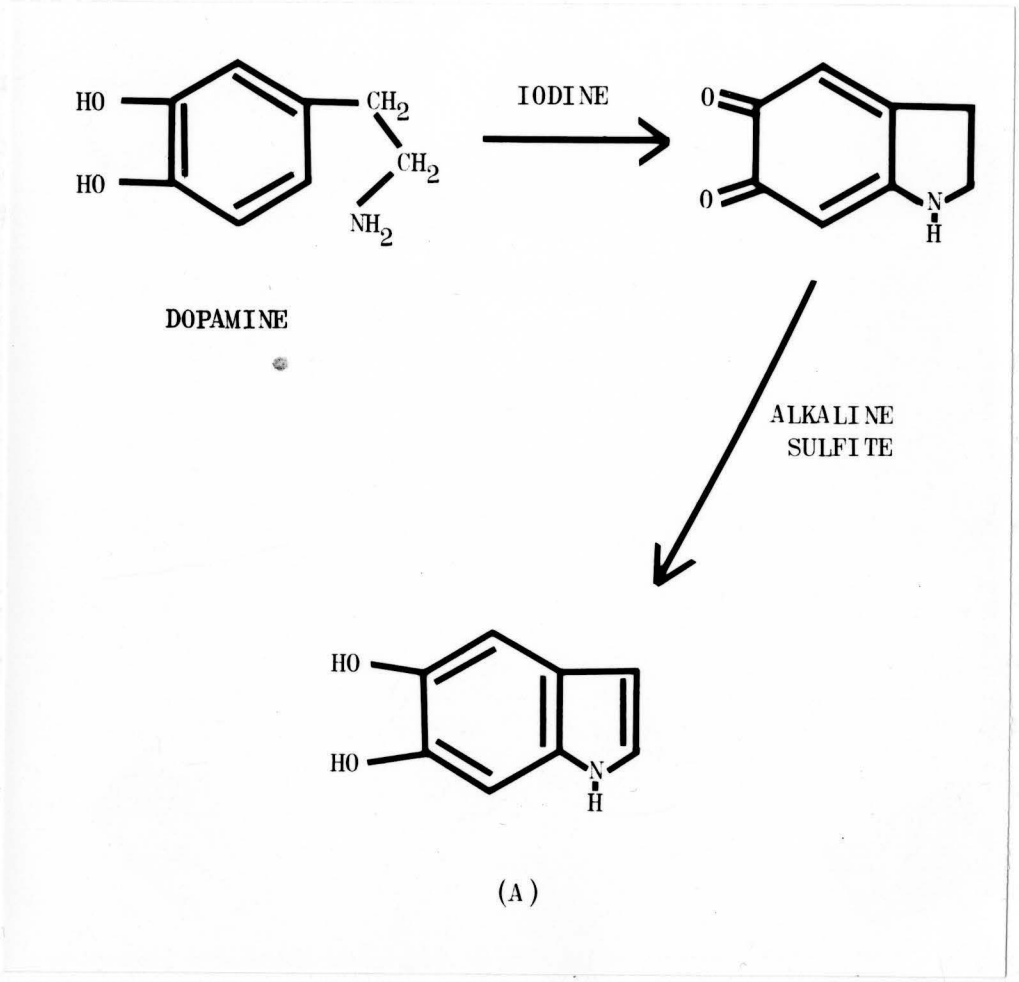


Figure 2. The conversion of dopamine to a fluorophore (A) via dihydroxyindole condensation.

acidified with 5 N hydrochloric acid. The DM fluorophore then fluoresces at a lower wavelength allowing a quantification free from extraneous catechol fluorescence.

Calculation of DM content in the tissues was performed by construction of a standard curve based on the standard solutions treated concomitantly with the samples (minus their respective blanks). Then, the dopamine content of any tissue was calculated as follows:

$B_x - A_x$ = the relative fluorescence of tube "B"

B_x = the reading for tube "x" in set B

A_x = the reading for tube "x" in set A

The amount (mcg.) of DM in the tissue sample "x" could then be ascertained from the standard curve. This amount was then multiplied by the tissue factor (the reciprocal of the tissue weight in grams) to give the concentration of DM in the tissue in micrograms/gram of wet tissue weight.

b. Dopamine, Serotonin, and Norepinephrine Determination in a Single Tissue Sample

The technique of Ansell and Beeson (1968) was used for simultaneous determinations of NE, 5-HT, and DM in the same brain sample (Figure 3). A tissue sample, weighing from 100-500 mg. was homogenized in 5 ml. of acid butanol using a glass Potter-Elvejhem homogenizing tube with a Teflon pestle (Kontes). One liter of acid butanol consists

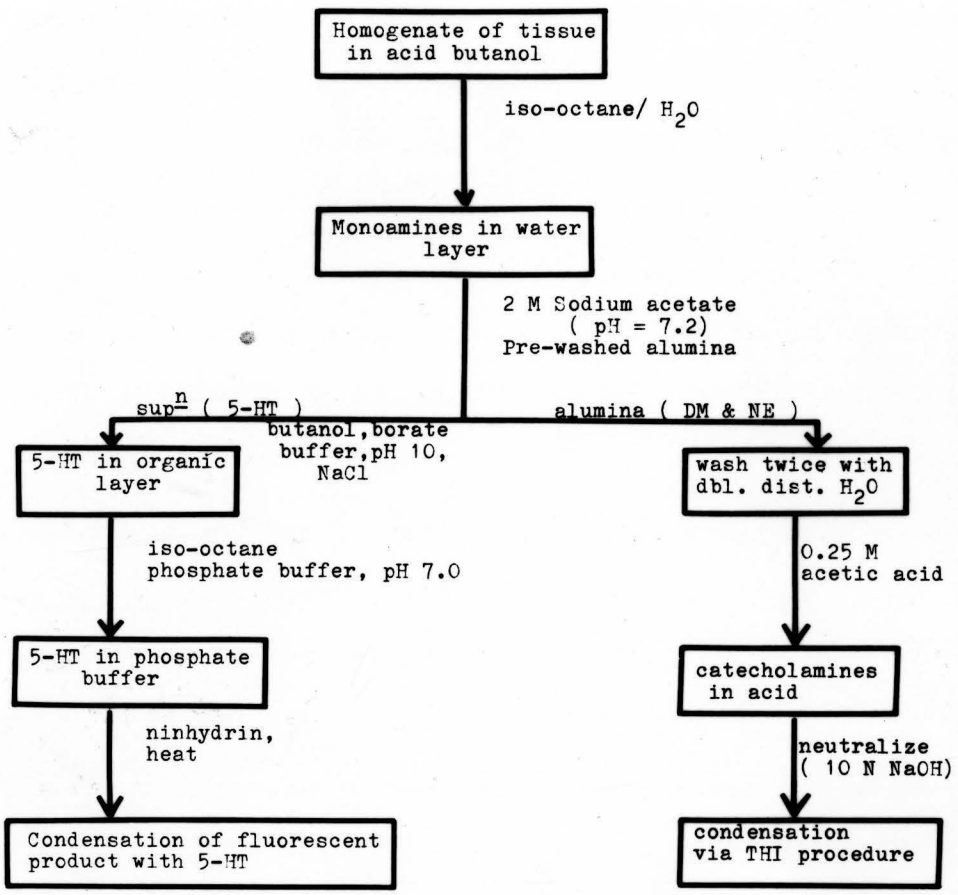


Figure 3. The Ansell and Beeson procedure (flow sheet). The supernatant is indicated by sup^{n}.

of 0.999 1. of washed butanol mixed with 0.85 ml. of concentrated hydrochloric acid, 1.0 gram of potassium metabisulfite as an anti-oxidant, and 0.1 gram of EDTA, disodium salt, used as a chelating agent for divalent cations. Homogenization was carried out with the base of the tube cooled in a beaker of ice to prevent monoamine degradation. All tissues were homogenized in 5 ml. of acid butanol with the exception of the cortex blank, which contained 400-500 mg. of cortical tissue homogenized in 10 ml. of acid butanol. This homogenate was split, and one portion was shaken with a 50 lambda portion of mixed standard solution containing 250 nanograms each of DM, NE, and 5-HT. All homogenates were centrifuged at 1000g for five minutes.

4.0 ml. of the supernatant butanol layer was then shaken in a 50 ml. glass-stoppered centrifuge tube with 10 ml. of 2,2,4-trimethylpentane (iso-octane) and 5.0 ml. of double distilled water. This melange was shaken at high speed on an Eberbach shaker for two minutes. This step transfers the monoamines from the butanol layer into the aqueous layer by solvent saturation. These vessels were centrifuged for two minutes at 1000g to separate the liquid layers. The upper (organic) phase was aspirated off with a Pasteur paper tip attached to a Tygon tube-faucet aspirator combination. 4.5 ml. of the aqueous phase was added to 1.0 ml. of 2M sodium acetate solution and 200 mg. of

pre-washed Woelm alumina in a 12 ml. glass-stoppered centrifuge tube (Kontes). Woelm alumina was pre washed with 1N hydrochloric acid followed by 35-40 rinses of distilled water to raise the pH to above 4.0, and then heated to 200° C. for two hours prior to storage in a dessicator. The pH of each sample tube was checked with a Beckman pH meter, and was adjusted to a range of 7.2-7.4 with 0.5N sodium hydroxide, when necessary.

This mixture was shaken for ten minutes, and then centrifuged for five minutes at 1500g. This step involves the adsorption of catecholamines onto the alumina, while the 5-HT remains in solution. Since catecholamines are unstable in alkaline media, the adsorption of catecholamines at pH 7.2 represents an improvement in methodology, which is possible because of the use of pre-washed alumina nd the absence of phosphate ions. The aqueous solution, containing 5-HT, was transferred to a 50 ml. glass-stoppered centrifuge tube containing 11.0 ml. of purified butanol. 1.0 ml. of 0.35 M borate buffer, pH 10, and 3.0 grams of sodium chloride. This melange was shaken for ten minutes on high speed and centrifuged for two minutes at 1000g. This step transfers the 5-HT from the salt-saturated, alkaline aqueous media into the organic phase. Then ten ml. of the organic phase were added to a 50 ml tube containing 15 ml. of iso-octane and 1.0 ml. of freshly prepared 0.05 M

phosphate buffer, pH 7.0. This mixture was shaken for two minutes at 1000g. This solvent saturation drove the 5-HT back into the aqueous media, and the upper organic layer was removed by aspiration.

A portion (1.2 ml.) of the aqueous phase from each tube was added to 0.1 ml. of fresh 0.1 M ninhydrin solution, and heated at 75° C. for thirty minutes. An unextracted standard (50 ng.), a blank, and a tissue blank were also treated similarly. After heating, the tubes were allowed to cool for a minimum of one hour. The fluorophore that is developed remains stable for several days. These samples were read in the Aminco-Bowman SPF at an excitation wavelength of 385 millimicrons (uncorrected) and at an emission wavelength of 495 millimicrons (uncorrected). Slit scheme number 5 is used for the quantification of the serotonin-ninhydrin cendensation product, which is linear in absorbance from 5-HT concentrations of 0.5-5000ng.

Meanwhile, the alumina was washed with 2 ml. of double distilled water, pH 7.0, by shaking for two minutes at high speed. The tubes were then eluted with 1.0 ml. of 0.25 M acetic acid. The tubes were shaken for fifteen minutes at high speed, and spun at 1500g for five minutes. The supernatant acid was transferred to an 18 x 150 mm. test tube with a Pasteur pipet. These samples were adjusted to

pH 6.50 by the addition of 10 N sodium hydroxide from a micrometer syringe. The pH was continuously monitored with a probe electrode and Beckman pH meter, while the tube was shaken on a table-top vortex mixer.

A 0.5 ml. portion of the adjusted supernatant was mixed with 50 microliters of 0.1 M EDTA in 1.0 M sodium acetate buffer, pH 6.50. EDTA is added to stabilize the catecholamine fluorophores, and it increases fluorescence in this procedure by 50-75%. Fifty microliters of 0.1 M iodine (in absolute ethanol) was added to begin the oxidation step of the hydroxyindole formation (Figure 2). This was followed exactly two minutes later by the addition of freshly prepared alkaline sulfite (0.1 ml.). Then, after two additional minutes, 0.1 ml. of 6 M acetic acid was added to each tube. The tubes were heated at 100° C. for two minutes and cooled rapidly to room temperature. One ml. of double distilled water was then added to each tube, and these samples were read immediately for NE fluorescence. This fluorescence was read at an excitation wavelength of 385 millimicrons (uncorrected) and at an emission wavelength of 485 millimicrons (uncorrected). These samples, as well as those of DM, were read at slit scheme number 2 in the Aminco-Bowman SPF. The samples were then transferred from the cuvette back into their respective tubes, and they were re-heated for four minutes at 100° C., to destroy the NE

fluorophore. To assure the absence of NE interference, the samples were assayed for DM on the following day. DM fluorescence is excited at a wavelength of 330 millimicrons and read at emission wavelength of 385 millimicrons (both uncorrected).

Amine content is calculated by comparison with the internal standard as described below:

$$\frac{R_x - B_x}{R_{std} - B_{std}} \times \frac{4}{4.5} \times 250 \times \frac{5}{4} \times \frac{1}{y} = \text{ng./gram of amine}$$

R_x = fluormetric reading of tube "x"

B_x = fluormetric reading of tissue blank

R_{std} = fluormetric reading of cortex standard

B_{std} = fluormetric reading of cortex blank

4/4.5 (250)= actual amount of amine extracted

5/4 = correction factor for the volume of butanol homogenate used

1/y = the tissue factor, where y is the wet tissue weight

This method has sensitivity for each of the amines as follows: 20 ng. for NE, 30 ng. for 5-HT, and 100 ng. for DM; where sensitivity is defined as that quantity of each amine which must be present in order to give a reading which is equal to twice that of its blank value. The recoveries with this method are in the range of 51 $\pm$ 8%.

c. 4-aminobutyric Acid (GABA)

GABA was extracted from tissues in ethanol (Baxter, 1961) and was assayed by the method of Jakoby (1962). The tissues were homogenized in a Potter-Elvejhem homogenizer with a Teflon pestle. The frozen sample was placed directly into the homogenizing vessel containing six ml. of ice-cold ethanol (80%). After homogenization, the pestle was rinsed into the tube with 2 ml. of 75% ethanol. The samples were then centrifuged at 1500g for ten minutes. The supernatant fluid was decanted into a twenty ml. beaker following centrifugation. The contents were evaporated to dryness under an infra-red lamp (Infra-dry cabinet, Precision Scientific Co.). Beakers were removed just at the point of complete evaporation to avoid the formation of a brown denaturation product, which increased blank values. One ml. of distilled water per 100 mg. of wet tissue was added to each beaker, and the resuspended extract was transferred to a 3 ml. polycarbonate centrifuge tube (11 x 77 mm.). These tubes were centrifuged at 20,000 x g @ 4° C. for thirty minutes on an IEC B-20 high speed refrigerated centrifuge. A 0.2 ml. aliquot of each supernatant was pipetted into a 1 ml. Pyrex beaker, and the fluid was evaporated at 50° C. <u>in vacuo</u> (20-25 psi.). The tubes were stored in a vacuum desicator until the enzymatic determinations were performed.

Determinations were made by adding 0.5 ml. of double distilled water to the dried contents in the 1 ml. beaker. The resuspended solution was quantitatively transferred to a microcuvette of 10 mm. light path, 1.25 ml. total volume (Sargent Chemical Co.). 0.4 ml. of a reagent mixture was then added to the microcuvette. This reagent mixture contained 0.05 mls. of 1.0 M Tris buffer, pH 7.9, 0.15 ml. of 1.0 M sodium sulfate solution, 0.05 ml. of 0.7% mercaptoethanol in Tris buffer, pH 7.9, 0.05 ml. of nicotine adenine dinucleotide phosphate (NADP), solution, 20 mg./ml., and 0.1 ml. of a solution of GABASE-CF, 50 mg./ml. (concentration was increased only when aging of the enzyme necessitated a greater amount of enzyme so that the reaction could be completed in ten minutes). At this point, a control absorbancy reading was recorded at 340 millimicrons on a Beckman DU spectrophotometer. 0.1 ml. of a 0.02 M alpha-ketoglutarate solution, pH 7.9, is added to initiate the reaction indicated in Figure 4. Absorbance readings were then taken every thirty seconds until maximum absorption was attained.

The Tris buffer stabilizes the system against any pH change due to the release of hydrogen ions. Sodium sulfate is used to prevent protein precipitation during the reaction. NADP and alpha-ketoglutarate initiate the reaction *per se*. GABASE-CF is a cell-free preparation of

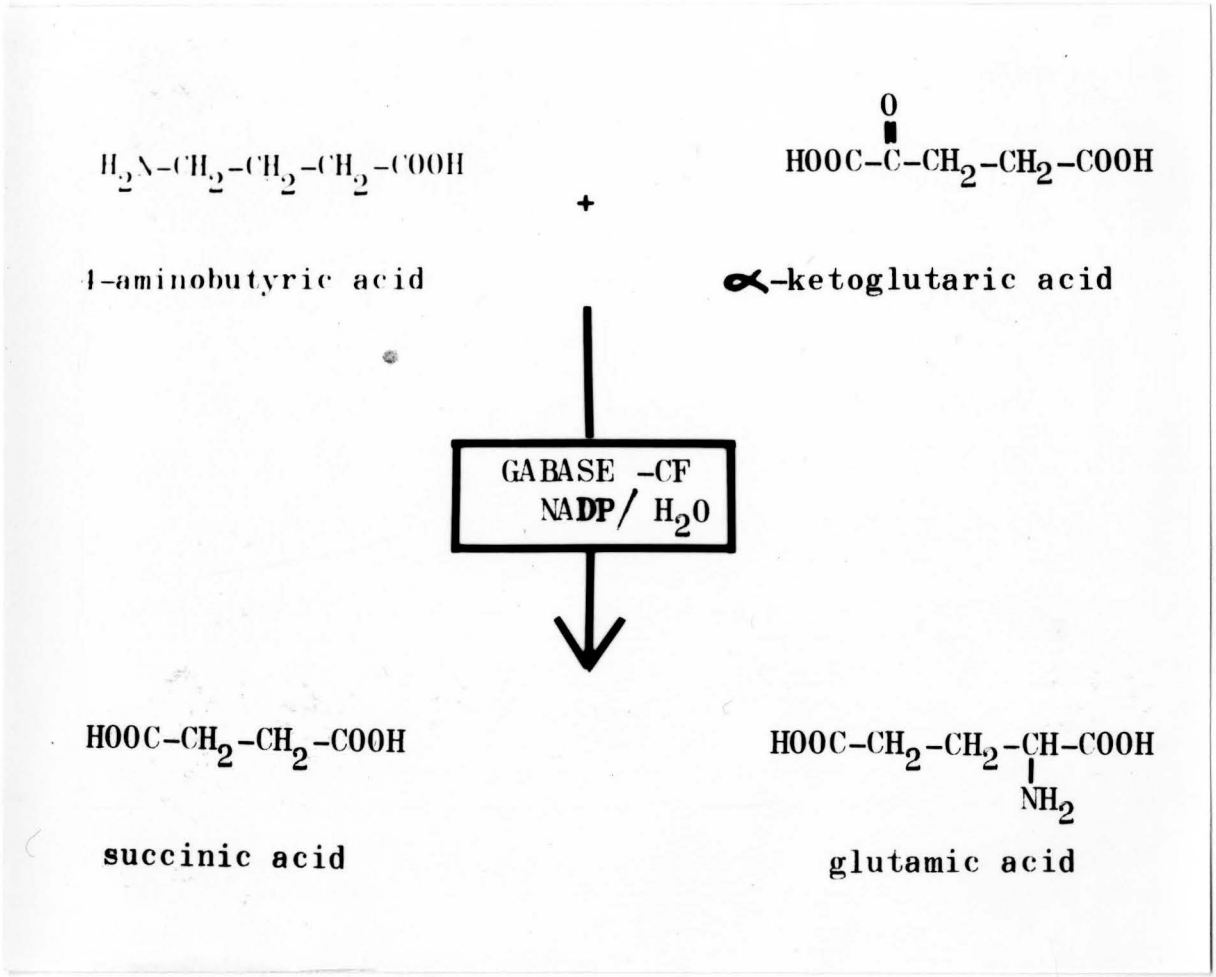


Figure 4. Reaction of GABA with GABASE-CF.

an enzyme, purified following extraction from Pseudomonas fluorescens (Worthington Biochemical Co.). This preparation is free from TPNH oxidase activity, and glutamate does not interfere in the reactions of this enzyme.

A standard curve of 4, 6, 8, and 10 micrograms standards was run with each group of samples, and these readings were used to plot a curve, which was linear in this range. The method was sensitive to 30 ng. of GABA in the tissue extract. The control reading for each sample was subtracted from the maximum absorbance change, and the actual value of GABA in the tissue was calculated as follows:

micrograms GABA $\times \frac{50}{103.1}$ = micromoles of GABA/gram WTW

50 = a constant based on resuspension of the tissue with 10 ml. water/gm. of tissue, and the subsequent use of 0.2 ml. of extract for the final reading

103.1 = the molecular weight of one micromole of GABA 50/103.1 is reduced to a constant, 0.48496, which is used when the calculations are made

d. Determination of Glycine

Glycine was quantified by modification of the colorimetric method of Aprison and Werman (1965). The tissues were homogenized in 5% TCA (in a volume equal to a 10% w/v homogenate) in a Potter-Elvejhem homogenizing tube (Kontes). The homogenate was transferred to a polycarbonate centrifuge tube (11 x 77 mm.), and samples were centrifuged at 23,000g at a temperature of 4° C. for twenty

minutes (IEC B-20 High Speed Refrigerated Centrifuge, No. 870 Head). 100 microliters of the supernatant from each sample were placed in 6 x 50 mm. Pyrex tubes. A similar quantity of 8 mcg./ml., 16 mcg./ml., and 32 mcg./ml. standard solutions was treated in the same manner. Forty microliters of a freshly prepared 7% sodium nitrite solution were added to each tube with the exception of a "glycine" blank (32 mcg./ml. glycine) and the tissue blanks. The blanks received distilled water in place of the sodium nitrite solution. The tubes were "buzzed" on a Beckman micromixer. Forty microliters of 1N sulfuric acid were added to each tube, and the tubes were "buzzed" again. Each tube was capped with a parafilm cover. A small pinhole was placed in each cover to allow for the escape of gaseous products, and the tubes were placed in a covered water bath at 100° C. for forty-five minutes. This step results in the diazotization of glycine to glycollic acid in the presence of nitrous acid and heat (the glycine content of the blank tubes is not converted, but remains as glycine throughout the procedure).

The tubes were cooled and centrifuged at 1500 rpm for five minutes to remove the condensation from the walls of the tube and concentrate it in the bottom of the tube. Forty lambdas from each tube were removed and placed in a Pyrex 10 x 75 mm. test tube. Five lambdas of a freshly

prepared 200% stannous chloride solution were added to each tube, and the tubes were "buzzed". This addition of stannous chloride results in the elimination of excess nitrite, which will otherwise interfere with the next reaction. 1.0 ml. of concentrated sulfuric acid, reagent grade, was added to each tube in order to convert the glycollic acid to formaldehyde. Then 20 microliters of a fresh 5% chromotropic acid solution were added to each tube in subdued light, and the tubes were "buzzed". Each tube was "buzzed" and placed in darkness immediately following this addition because chromotropic acid is extremely sensitive to light, and any extended exposure will disrupt the colorimetric reaction. All tubes were allowed to cool for forty-five minutes in the dark. Samples were then measured for their absorbance in a Coleman 6/10 spectrophotometer by using 10 x 75 mm. glass cuvettes. The chromophore is measured at 580 millimicrons (Figure 5).

Glycine concentration, in mcg./ml., for the original solution was calculated from the standard curve. Blanks were subtracted from their respective samples. Then, the concentration of glycine in mcg./ml. was multiplied by 10/75.1, and the result of this manipulation was equal to the concentration of glycine in the tissue in micromoles per gram of wet tissue weight. The number "10" is used as

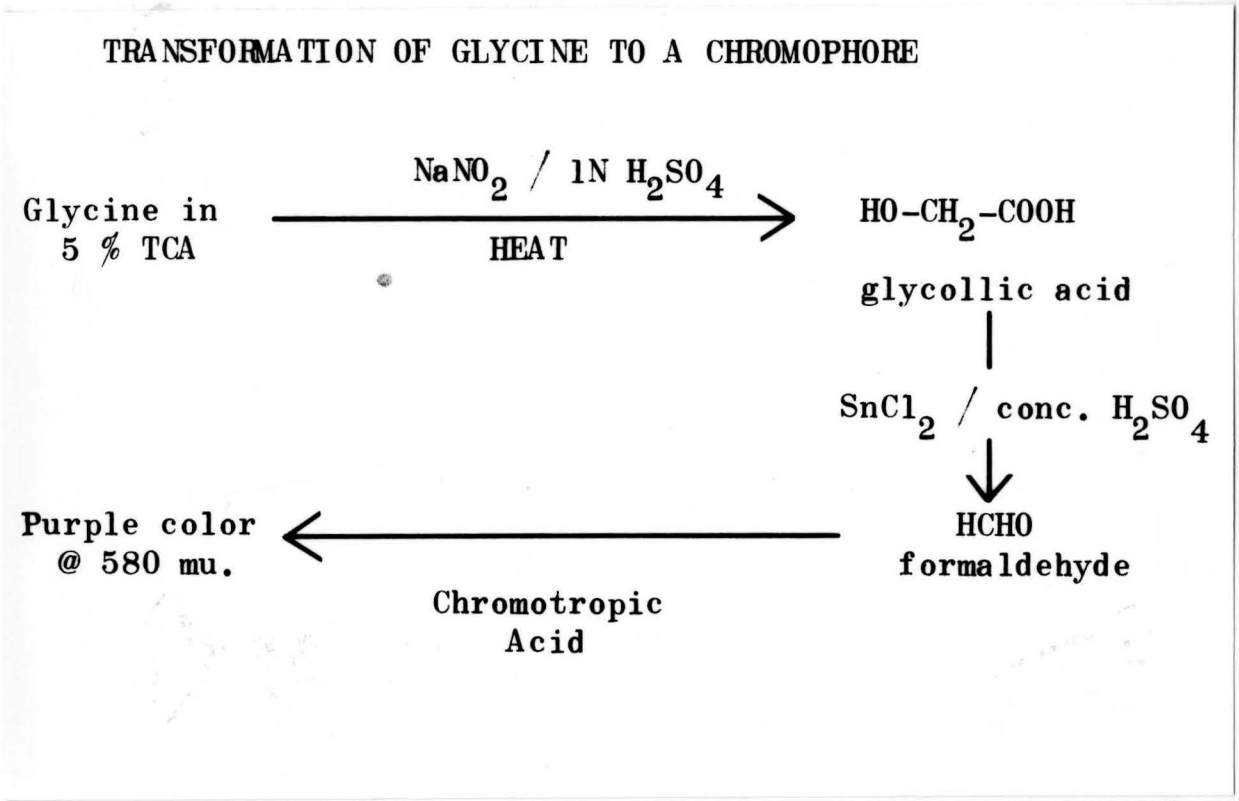


Figure 5. Transformation of glycine to a chromophore.

a multiplication factor because of the 10% dilution during homogenization. The figure "75.1" is the molecular weight of glycine. This method is highly specific, and does not suffer from the recovery losses associated with chromatographic techniques. It has been found to be sensitive to 2×10^{-8} moles of glycine.

B. Determination of Rectal Temperature Rhythm

Rectal temperature was monitored in a group of sixty male rats at three hour intervals over a 24 hour period. A Yellow Springs Telethermometer (Model no. 44 TA) equipped with a flexible thermister probe (YSI Model no. 42 SC) was used for these measurements. The probe was cleaned with water at 32° C. and dried after each temperature determination. This procedure allowed rapid equilibration to the actual animal temperature because the temperature of the probe approached that of the animal. The probe was lubricated with Mazola Corn Oil (Corn Products Co., New York) and inserted approximately five centimeters into the colon. The thermister probe was maintained in situ until the temperature reading remained constant for a period of 40-50 seconds. The probe was then removed, and the temperature recorded. Animals were handled in a gentle manner in order to avoid any excitement which could interfere with the temperature measurement.

C. Motor Activity Rhythms in Mice

Animals were adapted for four weeks to either a normal or reverse cycle. Six mice were then placed into individual photoactometer chambers with food and water available ad libitum. They were allowed to acclimate to this condition for at least twelve hours prior to activity monitoring. The photometer unit is shown in the photograph in Figure 6A and 6B. This cage has six photocells placed equidistantly around the perimeter of the cage. An infra-red light, located in the center of the unit, emits a continuous beam to the photocell; whenever that beam is broken, and activity count is recorded on a separate counting unit. The counters were placed outside of the animal quarters to prevent the clicking sound of the counters from affecting the activity of the mice. Animal activity was monitored for a sixty minute period, which was checked with a stop-watch to assure accurate timing of the counters. At the end of the 24-hour observation period, the availability of food and water was confirmed in each unit.

D. Measurement of Response to Maximal Electroshock (MES) and the Evaluation of the Effects of Various Convulsant and Anti-convulsant Drugs

1. MES Methodology

MES seizures were evoked in mice using a shock

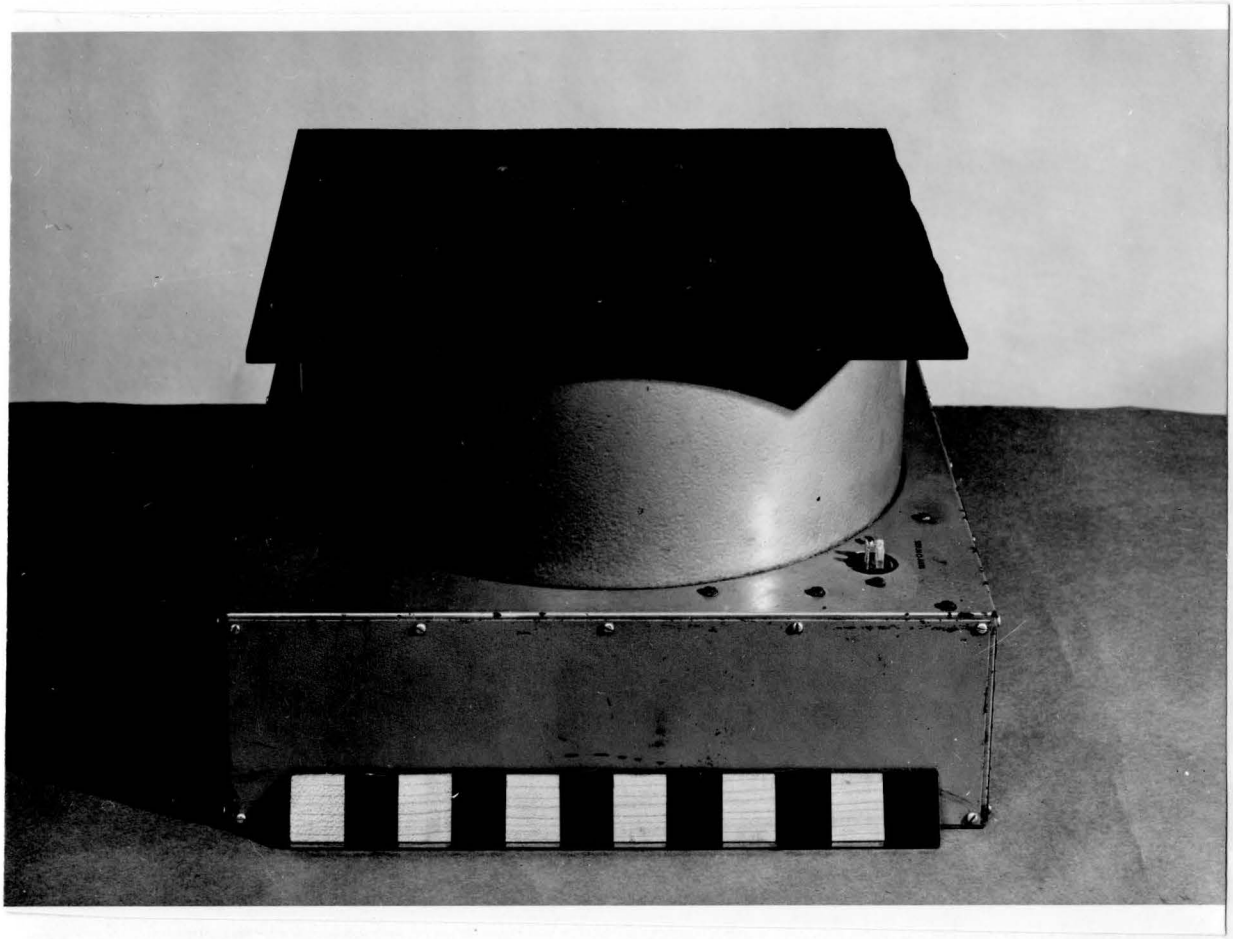


Figure 6A. Front view of photoactometer cage. The scale noted on the checkered ruler in the foreground of the picture is equal to one inch per division. A complete description of the apparatus is given in the text.

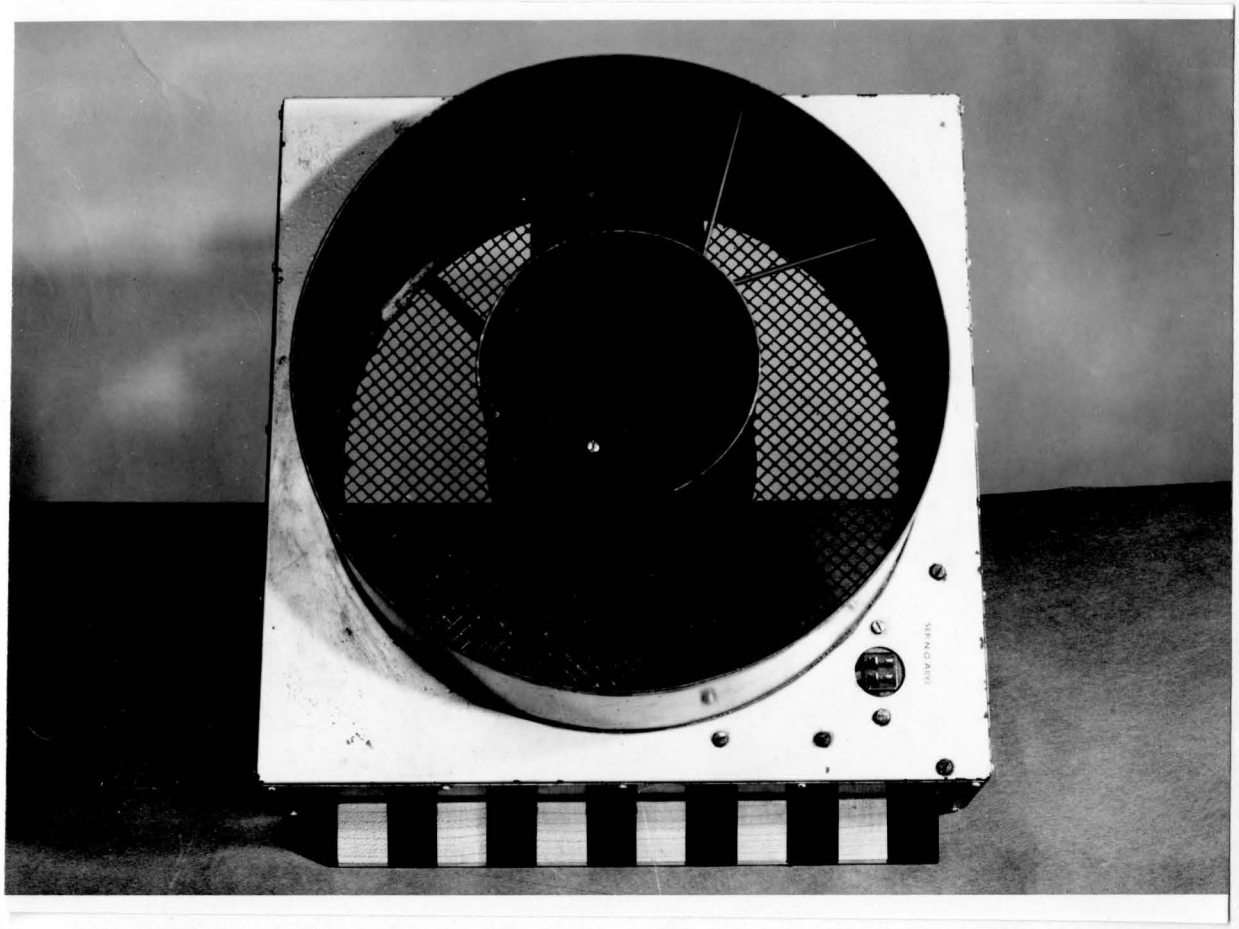


Figure 6B. Top view of photoactometer cage. The scale noted on the checkered ruler in the foreground of the picture is equal to one inch per division. A complete description of the apparatus is given in the text.

apparatus as described by Woodbury and Davenport (1952, cf. Figure 7). The shock was administered via corneal electrodes, which had been moistened with 0.9% saline solution. The stimulus parameters were current intensity of 50 ma. with a duration of 0.2 second as described by Swinyard et al., 1952. Three Cramer timers were arranged to start simultaneoulsy with each stimulus, and these timers were stopped following the various phases of the seizure (Figure 8).

A similar study was performed in rats that were adapted and shocked at the University of Nebraska College of Pharmacy. The adaptation lighting at the latter institution consisted of sunlight (through window glass) rather than Vita-lite. The MES technique was also similar to that previously described for mice except that the stimulus parameters were 150 ma. at 0.2 seconds.

Animals to be shocked were picked up and held by the scruff of the neck; their eyes placed in contact with the premoistened corneal electrodes, and the shock was delivered by the depression of a foot-switch. The components of MES seizure, as described by Toman et al. (1946), were measured on a temporal basis by stoping a timer precisely when each phase ended. It was noted that my reaction time was 0.15-0.20 second. This time was equal to the time of duration of the initial shock, so that all times given in

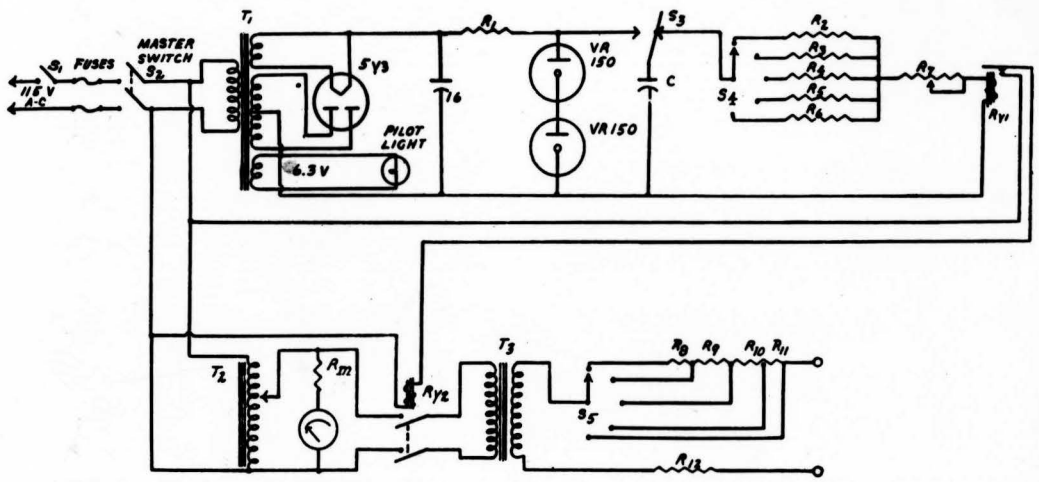


Figure 7. Woodbury-Davenport shock apparatus. (Copied with permission of the publisher, Arch. Intl. Pharmacodyn. 192, 100, 1952).

See next page for diagram legends.

Figure 7 diagram legends:

S_1	Interlock switch on cabinet door
S_2	Master switch
S_3	Push button to initiate timer; normally connected to timer
S_4	Timer switch 5 pole, single circuit wafer type
S_5	Current range switch, porcelain base, high voltage
T_1	Small replacement type transformer 350-0-350 volts
T_2	Variable autotransformer, 3 ampere capacity
T_3	Plate supply transformer, primary 110 volts, secondary 2000 volts, 400 ma.
C	Timer condensor, 5 mfd, 1000 volts
Ry_1	Sensitive relay, 10,000 ohm winding
Ry_2	Heavy duty, double pole relay or contactor, 110 volt coil
R_1	Adjust to give 25-30 ma. through regulator tubes; approximately 2,000 ohms, 10 watts
R_2-R_6	Select to give desired time of closure
R_7	Adjust to give slight changes in timing of all timing positions
R_8-R_{12}	Wire wound, 100 watt variable resistors with sliding taps. Adjust to give desired full scale current. The following values are approximate:

R8	120,000 ohms
R9	40,000 ohms
R10	20,000 ohms
R11	16,000 ohms
R12	4,000 ohms

Meter: (Indicated by circle-enclosed arrow in lower left part of diagram) Any meter capable of reading rms-a-c volts; scale may be hand-calibrated to give desired current ranges.

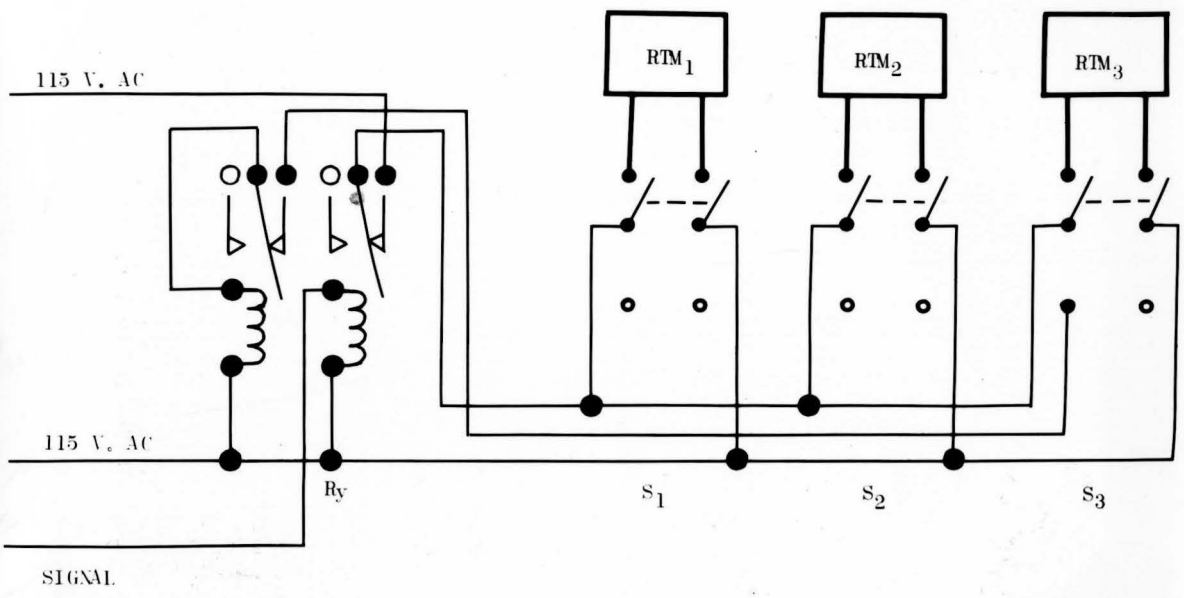


Figure 8. Electrical diagram of timing apparatus used for measurement of seizure component times.

the text are actually timed from the end of shock administration. The times recorded were: 1) the time to the beginning of the tonic extensor phase, or extensor latency time; 2) the time to the end of the tonic extensor phase; and 3) the time to the end of the clonic phase prior to post-ictal depression.

Animals were used only once, and discarded following MES treatment. MES treatment and the testing of anticonvulsant drug potencies were evaluated at the two time points, which appeared from our biochemical studies to represent the peak and trough of CNS biogenic amine levels.

2. Pharmacological Studies and Drug Protocol

a. Acute Toxicity of Convulsant Drugs

The acute toxicity of convulsant drugs in mice was determined at six hour intervals for a period of 24 hours. The animals were conditioned to a normal light cycle three to four weeks prior to this study. Two to three groups of 9 animals each were injected I.P. with different doses of the test drug. After injection, the mice were placed in individual cages to avoid interaction with each other. The drugs used are potent central stimulants, and the movement or touch of one animal could trigger a seizure in an adjacent animal if aggregate housing was used. The toxic effects upon the animals were evaluated over a six hour

period, and the number of deaths in that time interval was recorded.

Allylglycine (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 0.9% saline prior to use. The concentration was adjusted to allow the injection of 0.1 ml. per 10 grams of animal weight. Strychnine sulfate (Merck, Rahway, N.J.) was prepared in a concentrated stock solution, so that it could be diluted 1:100 prior to use. It was administered by means of a 1.0 ml. tuberculin syringe in volumes of 0.01 ml. per gram of animal weight. The stock solutions were refrigerated when not in use.

The LD50 doses of these drugs were calculated as described in the statistics section (vide infra).

b. Protective Doses of the Anti-convulsant Drugs

The PD50 (median protective dose) against MES of each of the following drugs was determined during anticipated peaks and troughs of biogenic amine levels: diphenylhydantoin (Parke, Davis & Co., Detroit, Mich.), phenobarbital sodium (Merck, Rahway, N.J.), meprobamate (Wyeth Laboratories, Philadelphia, Pa.), acetazolamide (Lederle Laboratories, Pearl River, N.Y.), and aminooxyacetic acid hemihydrochloride (Aldrich Chemical Co., Milwaukee, Wisc.). Phenobarbital sodium and aminooxyacetic acid (AOAA) were administered in 0.9% saline

solution. Diphenylhydantoin, acetazolamide, and meprobamate were dispersed in a 5% acacia suspension. When necessary, the pH of solutions was adjusted to a range of 7.0-8.0 as determined with narrow range Hydrion test paper. Drug concentrations were adjusted so that 0.1 ml. of fluid would be injected for each 10 grams (mouse) or 100 grams (rat) of animal body weight.

Groups of 9 mice or 7 rats each were injected I.P. with a series of predetermined doses of the test drug, such that the low dose would produce protection below, and the high dose above, the anticipated PD50 value. In addition, a group of ten saline control mice, a group of ten acacia-treated control mice, or 7 saline-treated rats were tested with MES at the chosen time intervals. These mice were specially selected for a uniform body weight of 34 gm. Rats weighed in the range of 275-290 gm. The volume of saline or gum acacia administered was similar to that of the drug-treated animals. One hour after drug or control media, the animals were subjected to MES stimulation (vide supra). The timing of the seizure components of the controls was recorded as well as the timing of those seizure phases which occurred in drug-treated animals.

PD50's were calculated for the various drugs as described in Chapter III E.

c. Neurological Evaluations of Motor and Reflex Activity of Rodents

Following injection of the anti-convulsant agents, animals were evaluated as follows: 1) observed activity - a visual observation of the animal's alertness, interaction with his environment, and a comparison to control animals with regard to frequency of movement; 2) balance - a test of the animal's ability to balance on the edge of a cage for at least five seconds (normal animals can perform this task readily); 3) reflex activity - an evaluation of the animal's capability to return his hindlimb to a surface following its withdrawal from that surface (replacement is rapidly performed by a neurologically sound animal, but not by a neurologically deficient one).

The summary of these evaluations is described in the results section.

E. Statistical Analysis of the Data

1. Calculation of Standard Deviation, Standard Error of The Mean, and Mean Values

The calculation of standard deviation (S), standard error (Sy), and arithmetic mean ($\bar{Y}$) were all performed on an SCM Cogito 1016 PR computer programmed with a Iota 1 tape input accessory. The formulae used for calculation of these values are described below.

$$\text{Arithmetic mean} = \frac{\text{sum of the independent values}}{\text{the number of entries}} = \frac{\Sigma y}{n}$$

$$\text{Variance } (S^2) = \frac{\Sigma(y-Y)^2}{n-1} \quad \text{where } n-1 \text{ degrees of freedom and } y = \text{each entry}$$

$$\text{Standard deviation } (S) = \sqrt{S^2}$$

$$\text{Standard error of the mean} = \sqrt{\frac{S^2}{n}}$$

2. Calculation of t

The t test for non-paired data was used to evaluate the level of significance of compared samples. The data was entered into the SCM computer and a programmed formula was used to calculate t. The formula is represented below.

$$t = \frac{\bar{Y}_1 - \bar{Y}_2}{\sqrt{\frac{n_1 S_1^2 + n_2 S_2^2}{n_1 + n_2 - 2}\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

3. Calculation of PD50 and LD50

The statistical analysis of these parameters and their respective calculations were performed according to the method of Litchfield and Wilcoxon (1949). The method is as follows:

1) an odd number of animals is always used to insure that the 50% value can be interpolated from the data.

2) the number of animals protected, or the number of deaths, at each dose level is converted to a percentage of the total amount of animals used at that dose. This is then converted to a probit value. A probit value of 5.000 represents the median lethal dose.

3) X_1 = high dose (mg./Kg.); X_2 = low dose (mg./Kg.)

4) Y_1 = mortality rate, or protection rate from X_1
   Y_2 = mortality rate, or protection rate from Y_2

5) The slope, b, = $\frac{Y_1 - Y_2}{X_1 - X_2}$ $\frac{\text{(Probit)}}{\text{(log)}}$

6) Standard Deviation (S) = 1/b $(\sqrt{n/2})$ where n = the total number of animals per dose.

7) Log dose = $X_1 + \frac{(5.000 - Y_1)}{b}$

8) The anti-log of the log dose is equal to the LD50 or the PD50 in mg./Kg.

A specific example of this procedure (Friedman, 1966) is shown below:

Dose	mg./Kg.	Log Dose	Mortality	Percent	Probit
X_1	100	2.00000	Y_1 = 7/9	77.8	5.765
X_2	75	1.87506	Y_2 = 2/9	22.2	4.235

$$b = \frac{Y_1 - Y_2}{X_1 - X_2} = \frac{5.765 - 4.235}{2.00000 - 1.87506} = 12.1456$$

$$S = 1/b\ (\sqrt{n/2}) = 1/12.2456\ (\sqrt{9/2}) = 1/25.9774 = 0.03849$$

$$\text{Log dose} = X_1 + 5.000 - Y_1/b = 2.00000 + 5.000 - \frac{5.765}{12.2456}$$
$$= 2.00000 - 0.06247 = 1.93753$$

Antilog of 1.93753 = 86.60

1.939753 + 0.03849 = 1.97602
1.939753 - 0.03849 = 1.89904

The antilog of 1.97602 = 90.63
The antilog of 1.89904 = 79.26
Difference = 15.37

15.37/2 = 7.685, or a standard deviation of 7.685 mg./Kg.

Therefore, the LD50 = 86.60 ± 7.685 mg./Kg.

The same method is also used for the calculation of the PD50 of the anti-epileptic drugs used.

4. Analysis of Variance

This test of significance was also used. The methodology and data are noted in Appendix C.

CHAPTER IV
RESULTS

A. Fluctuations of Body Temperature and Motor Activity

1. Rectal Temperature

The rectal temperature of sixty rats monitored every three hours for a twenty-four hour period is indicated in Figure 9. Average temperature of these nocturnally-active animals ranges from 37.95 ± 0.35° C. in the dark phase to 37.25 ± 0.25° C. during the light phase. The peak temperature of 38.3 ± 0.3 is noted at 2400 hours and a trough temperature of 37.1 ± 0.3 at 0900 hours. The curve shows the characteristic pattern of this parameter with a sharp increase in temperature following awakening. This increase is maintained throughout most of the dark phase, and the temperature decreases sharply as the light phase approaches.

Points recorded on the curve at the following times were found to be significantly different ($P < 0.001$) from each other when compared by the Student t-test: 0300 hours vs. all points; 0600 hours vs. both 0900 hours and 1200 hours; 0900 hours as compared with all points other than 1200 hours; 1200 hours vs. 2100 hours; 2400 hours as compared with all other points. The peak value is statistically significant with regard to all other values; and the trough is statistically significant with regard to all points on the curve except that at 1200 hours. Comparisons of all temperature values recorded in the light phase with all those

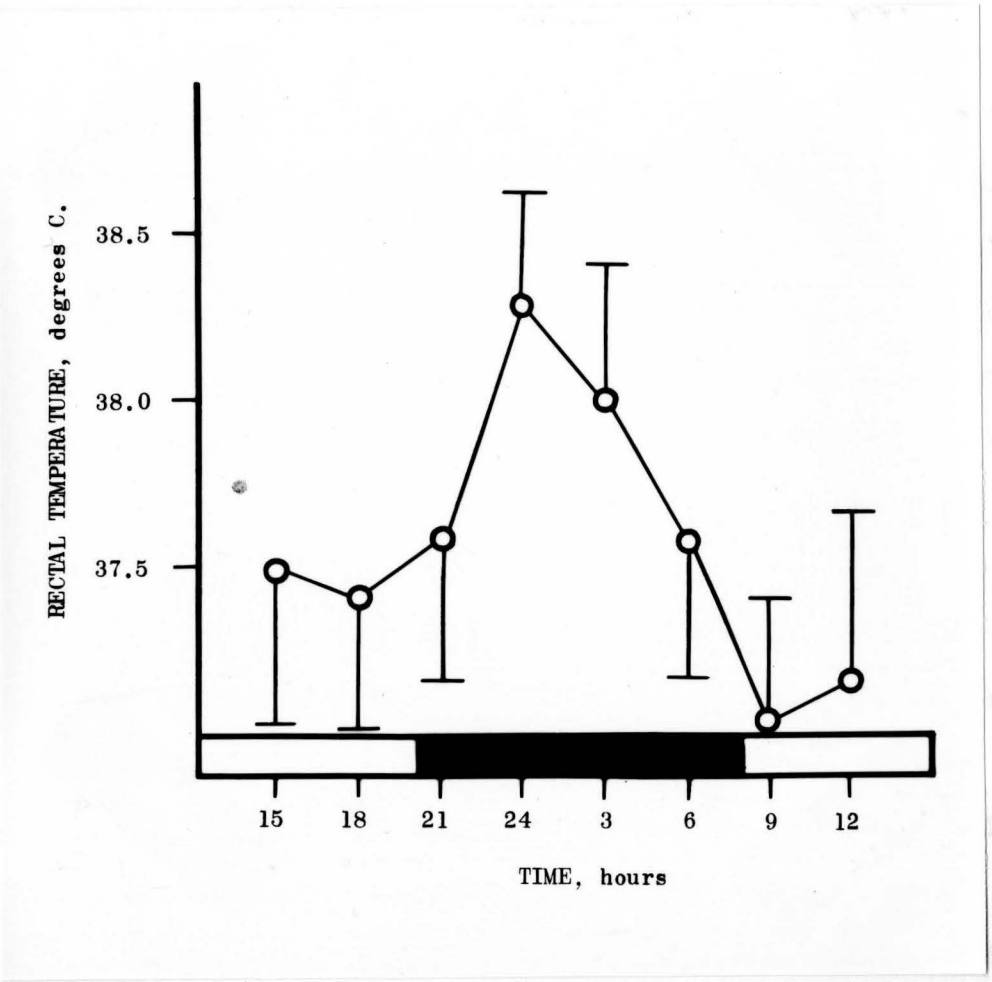


Figure 9. The circadian pattern for rectal temperature in rats. Each clear circle indicates the average temperature of a group of sixty rats. The ordinate represents rectal temperature in degrees C. The black bar on the abscissa indicates darkness. Standard deviations are indicated by the vertical brackets.

of the dark phase also are statistically significant.

2. Motor Activity

The motor activity curves for both "normal" and "reverse" cycled mice are indicated in Figure 10. The activity is expressed as a percent of the twenty-four hour average because of the variability of the rate of motor activity in the individual animals. This procedure for expressing data allows one to compare the activity of each animal with that of others whose rate of activity might differ markedly.

Motor activity rises sharply following the onset of the dark phase. Both mice and rats are considered to be nocturnal animals, and this curve is characteristic for these species. Figure 10 clearly shows that reversal of the light program reverses the motor activity rhythm.

Peak motor activity on the normal cycle occurs at 2400 hours (266%), and trough activity at 1800 hours (23%). The 2400 hour value is significantly greater ($P < 0.001$) than all other curve values. Peak motor activity on the reverse cycle also occurs during the dark phase at 1200 hours (226%) while a trough occurs at 2400 hours (41%). This peak value is also significantly greater ($P < 0.001$) than all other values of the curve.

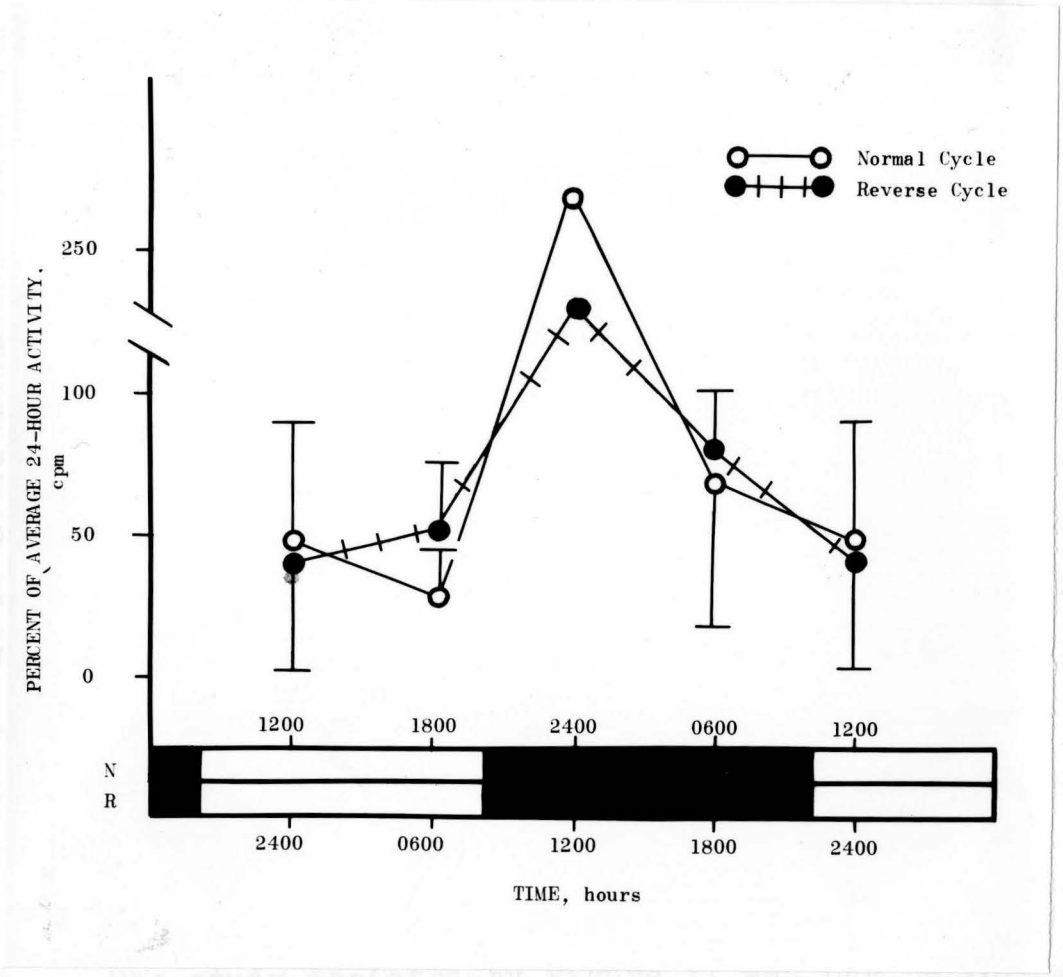


Figure 10. The motor activity of mice on "normal" and "reverse" cycles. The ordinate indicates the percent of average 24 hour activity. The black bar on the abscissa indicates the period of darkness. Time in hours is indicated for both "normal" (N) and "reverse" (R) cycles. The open circles represent N animals, and the filled circles the R animals. The vertical brackets denote the standard deviation (n = 6 animals per point); the standard deviation of the 2400 N and 1200 R points are 266 ± 57 and 226 ± 46 respectively.

B. Fluctuations in the Levels of Putative Inhibitory Transmitters

1. Dopamine

The dopamine (DM) levels of the corpus striatum of "normal" cycle rats are indicated in Figure 11. Levels rise during the dark phase to a peak at 0300 hours. Thereafter, they decrease steadily as the light phase approaches and finally trough at 1500 hours. The DM concentrations range from 3.46 $\pm$ 0.08 micrograms/gram of wet tissue weight (WTW) to 6.82 $\pm$ 0.24 mcg./gm. WTW. The peak value is significantly different ($P<0.05$) from both the 1500 hour point and the 1800 hour point. The trough is also significantly different from the 0300 and 0600 hour values ($P<0.05$).

The study depicted by Figure 11 was one performed in December, 1968, and the DM levels (assayed by the Sourkes and Murphey technique) were found to be much lower than those of a subsequent "reverse" cycle study (for discussion, see section C-1 of this chapter). DM levels were determined again in "normal" cycle animals in June, 1970. This study (Figure 12) includes measurement of DM levels in the upper and lower brainstem as well as in the corpus striatum (assayed by the Ansell and Beeson procedure).

DM levels in the corpus striatum peaked during the

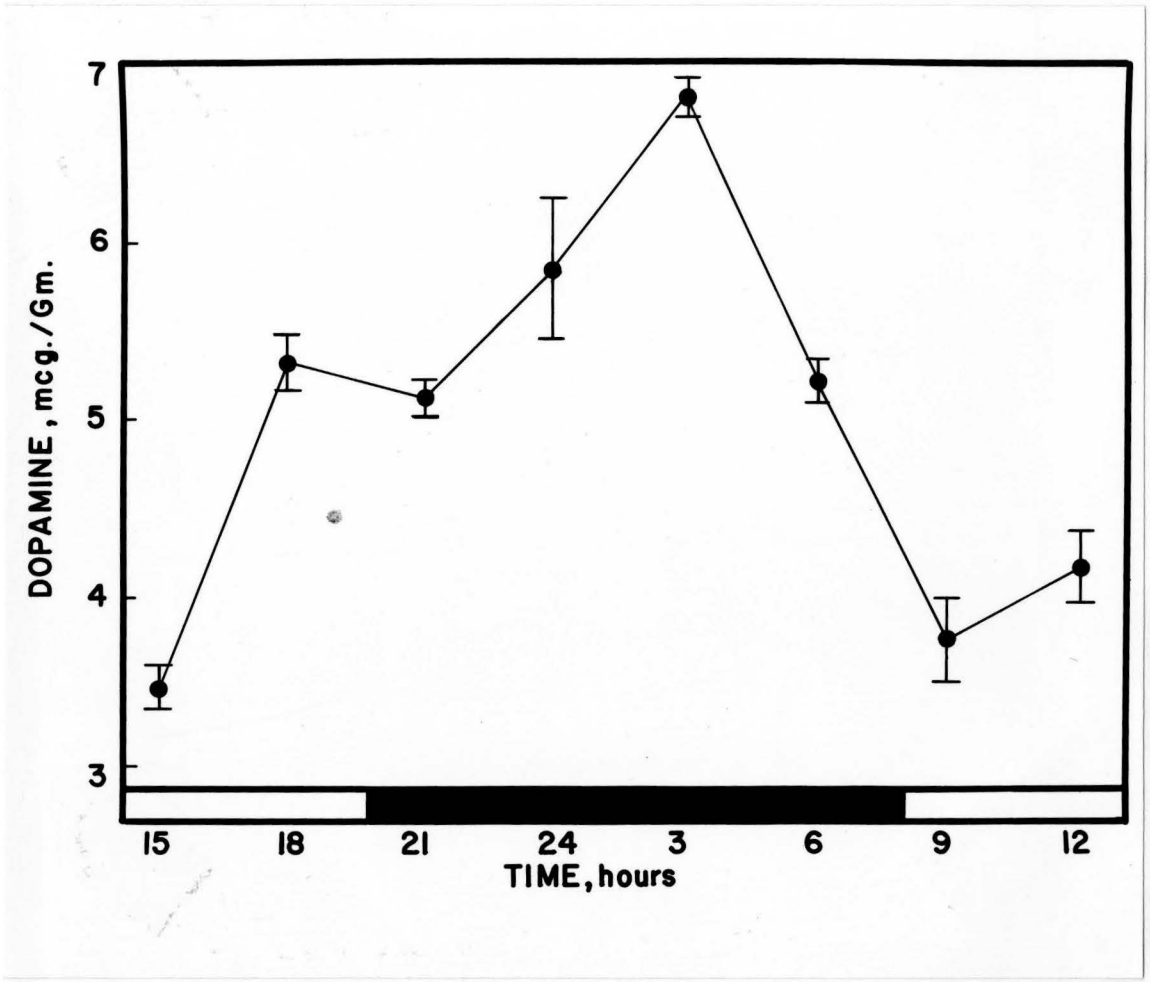


Figure 11. The circadian pattern for dopamine levels in the corpus striatum of the rat (December). The ordinate indicates the DM level in tissue in mcg./gm. WTW. Time is indicated on the abscissa. The black bar on the abscissa indicates the dark phase of the photoperiod. Vertical brackets indicate the standard error of the mean for the respective values (n = 6 animals per point).

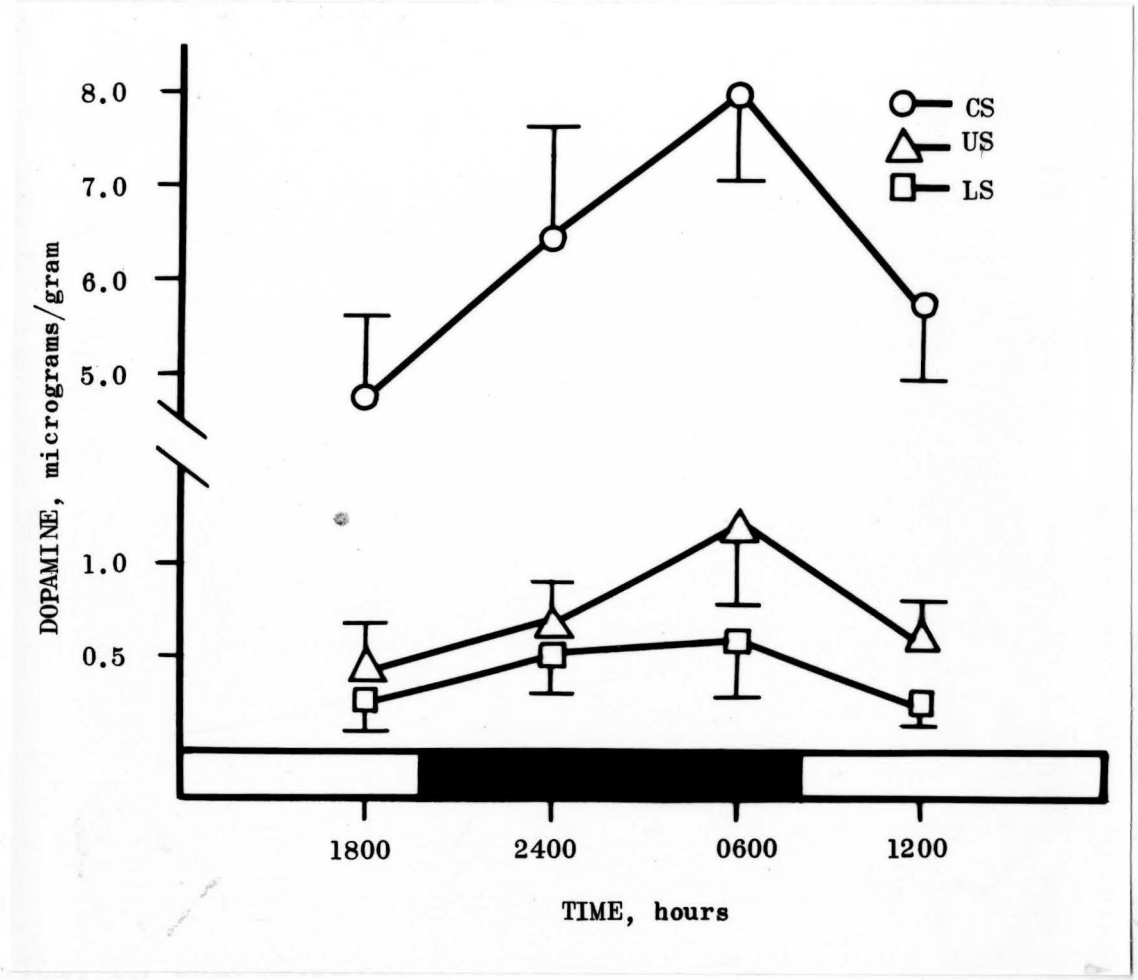


Figure 12. Dopamine levels in the rat corpus striatum (CS), upper brainstem (US), and lower brainstem (LS) in a 24-hour period (June). The ordinate represents the level of DM in mcg./gm. WTW. Time is represented on the abscissa. The black bar on the abscissa indicates the dark phase of the photoperiod. The vertical brackets indicate the standard deviation of the respective levels (n = 6 animals per point).

dark phase as in the previous study; however, this study was conducted with four sample periods/24 hours instead of 8/24 hours as in the former study. The peak DM levels were obtained at 0600 hours, and the trough value at 1800 hours. DM levels of the corpus striatum ranged from 4.81 ± 0.71 mcg./gm. WTW (1800 hours) to 7.99 ± 0.87 mcg./gm. WTW (0600 hours). The peak value was significantly greater ($P<0.01$) than both DM values of the light phase.

The DM levels of the upper brainstem also peak during the dark phase at 0600 hours, and the trough value, as in the case of the corpus striatum, was noted at 1800 hours. The DM levels varied from 0.46 ± 0.16 mcg./gm. WTW (1800 hours) to 1.23 ± 0.41 mcg./gm. WTW (0600 hours). The peak value was found to differ significantly ($P<0.05$) from both values obtained during the light phase. In the lower brainstem, DM concentrations were found to have a similar pattern to those of the corpus striatum and the upper brainstem; however, the trough levels occurred at 1200 hours. The 1200 hour value was significantly lower ($P<0.05$) than both values obtained during the dark phase. Levels in this tissue ranged from 0.25 ± 0.11 mcg./gm. WTW (1200 hours) to 0.54 ± 0.23 mcg./ gm. WTW (0600 hours).

2. Norepinephrine

The levels of NE obtained during a 24-hour period,

are indicated in Figure 13. NE and DM levels were sampled in the same tissues in order that the levels of these two amines could be compared.

NE levels in the corpus striatum were maximal during the dark phase with a peak at 0600 hours. A significant difference ($P < 0.01$) was obtained when the peak value was compared with values obtained for either of the points occurring during the light phase. The level of NE at 2400 hours (0.24 ± 0.07 mcg./gm. WTW) was significantly higher ($P < 0.01$) when compared with the levels of samples obtained for either point during the light phase. The levels ranged from 0.06 ± 0.02 mcg./gm. (1800 hours) to 0.24 ± 0.07 mcg./gm. WTW (0600 hours).

The pattern of NE levels of the upper brainstem was similar to that of the corpus striatum. Maximal levels were noted at 0600 hours with a trough at 1200 hours. Each point of the dark phase was greater and significantly different ($P < 0.01$) from each point of the light phase. Levels varied from a trough value of 0.95 ± 0.24 mcg./gm. WTW (1200 hours) to a peak value of 1.83 ± 0.34 mcg./gm. WTW (0600 hours).

NE concentrations in the lower brainstem exhibited a unimodal pattern; however, in this case, the peak levels were noted at 2400 hours and a statistically significant

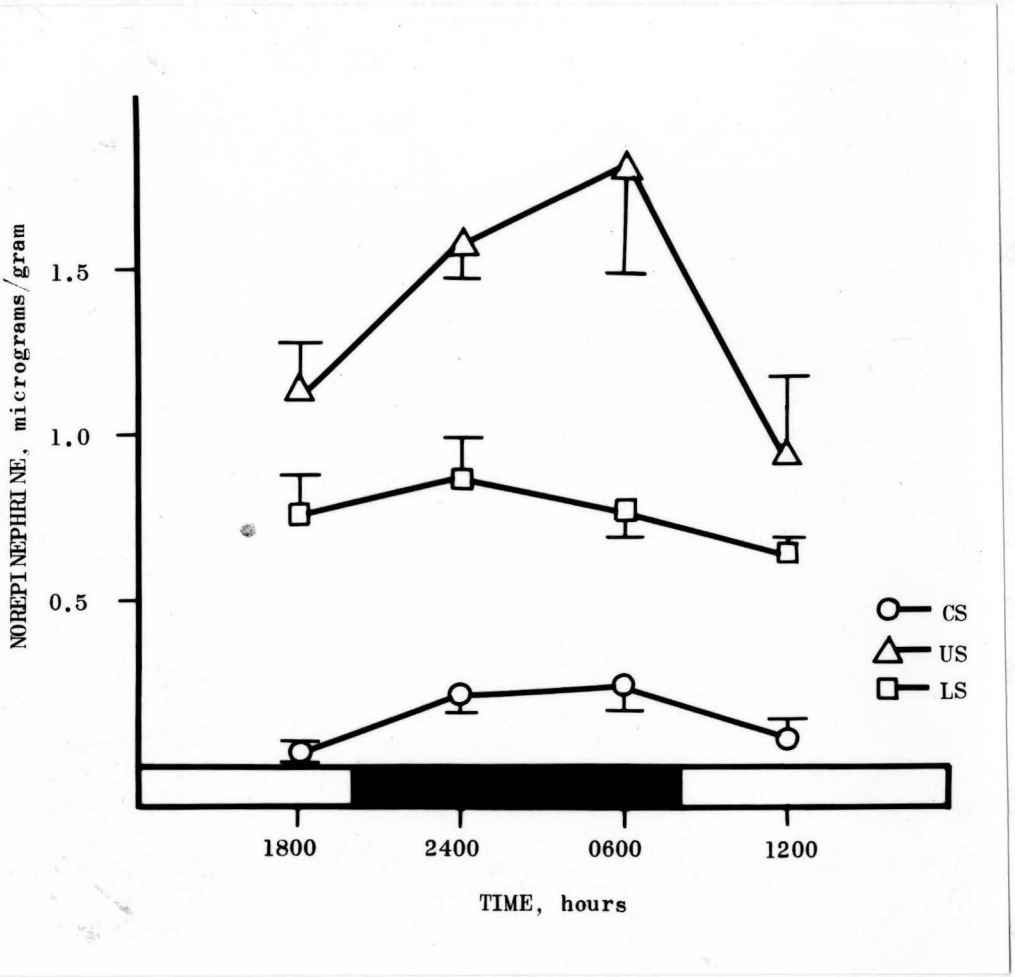


Figure 13. Norepinephrine levels in the rat corpus striatum (CS), upper brainstem (US), and lower brainstem (LS) in a 24-hour period. The ordinate indicates the level of NE in mcg./gm. WTW. Time is noted on the abscissa. The black bar on the abscissa indicates the dark phase of the photoperiod. The vertical brackets indicate the standard deviation of the respective levels (n = 6 animals per point).

($P < 0.01$) trough was obtained at 1200 hours. The concentrations varied from a trough value of 0.64 ± 0.03 mcg./gm. WTW (1200 hours) to a peak value of 0.88 ± 0.12 mcg./gm. WTW (2400 hours).

It is apparent in the tissues examined that DM and NE level patterns are similar.

3. Serotonin

The circadian patterns for 5-HT levels in the corpus striatum, upper brainstem, and lower brainstem are depicted in Figure 14. 5-HT levels of the corpus striatum follow a pattern inverse to those of the catecholamines. This pattern is marked by a trough in the dark phase: both points of this phase are significantly different ($P < 0.05$) from the peak value. 5-HT levels in the corpus striatum ranged from 0.46 ± 0.15 mcg./gm. WTW (2400 hours) to 0.94 ± 0.30 mcg./gm. WTW (1800 hours).

A similar pattern for 5-HT levels was noted in the upper brainstem. Levels varied in a circadian fashion with a peak value at 1800 hours and a trough value at 0600 hours. Values obtained for both points of the dark phase were significantly different ($P < 0.05$) from the peak value. Levels ranged from 0.65 ± 0.16 mcg./gm. WTW (0600 hours) to 0.93 ± 0.14 mcg./gm. WTW (1800 hours).

The pattern of 5-HT levels in the lower brainstem

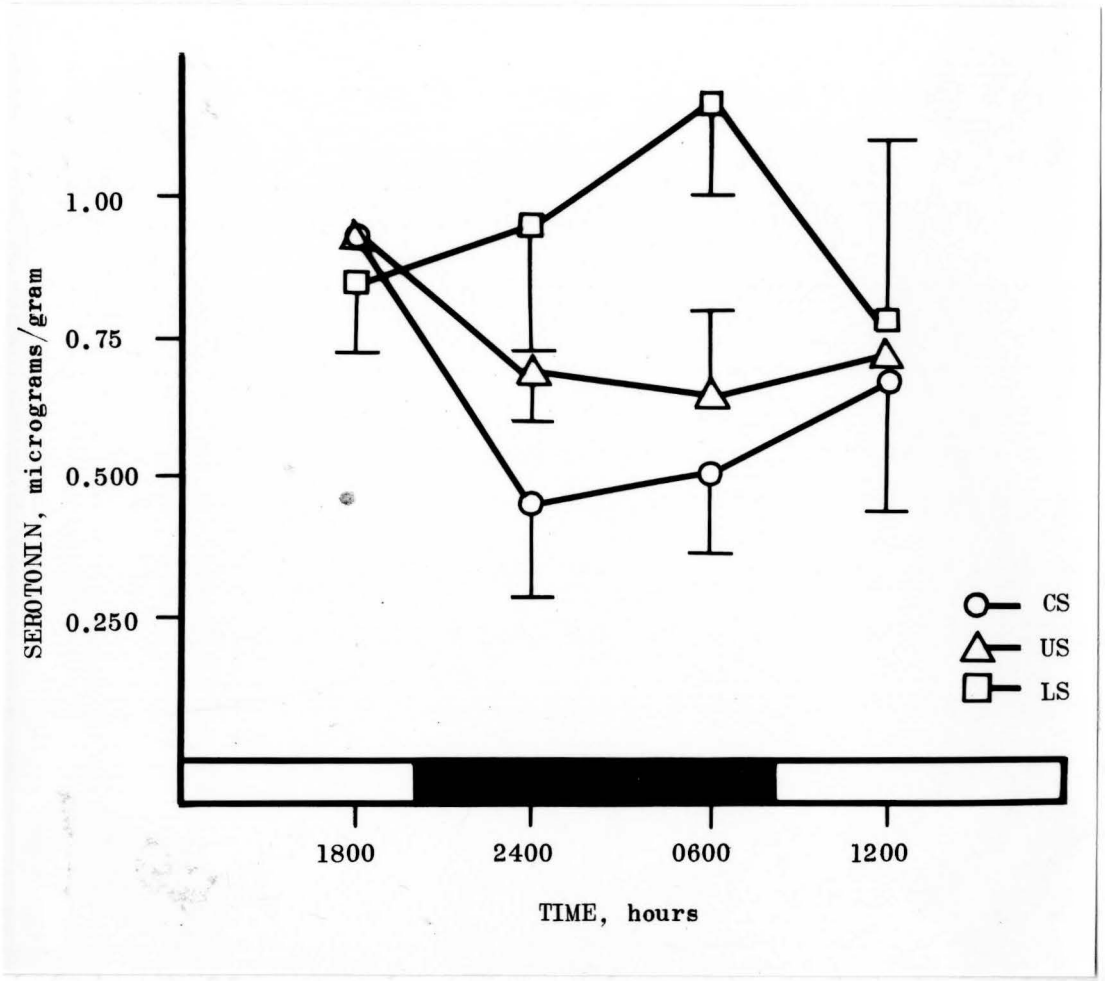


Figure 14. Serotonin levels in the rat corpus striatum (CS), upper brainstem (US), and lower brainstem (LS) over a 24-hour period. The ordinate indicates the tissue level of 5-HT in mcg./gm. WTW. Time is noted on the abscissa. The black bar on the abscissa indicates the dark phase of the photoperiod. The vertical brackets indicate the standard deviation (n = 6 animals per point).

differs markedly from those described above for this amine. A unimodal rhythm was obtained with a peak at 0600 hours and a trough value at 1200 hours. The peak value was significantly different ($P<0.05$) from either of the points in the light phase. The concentrations varied from 0.81 $\pm$ 0.26 mcg./gm. WTW (1200 hours) to 1.25 $\pm$ 0.26 mcg./gm. WTW (0600 hours).

4. 4-aminobutyric Acid (GABA)

The GABA patterns in the cortex and cerebellum are indicated in Figure 15. The cortical rhythm peaks during the dark phase at 0600 hours and troughs in the light phase at 1200 hours. The significant difference between these two points is only borderline ($P<0.10$). The study represented in Figure 15 was performed in June, 1970. A similar pattern was obtained in a study performed in March, 1969 (cf. Appendix A). However, the levels were proportionately lower (perhaps because GABA levels exhibit seasonal alteration). Cortical GABA levels varied from a trough value of 2.68 $\pm$ 0.33 mcM./gm. WTW (1200 hours) to a peak of 3.15 $\pm$ 0.45 mcM./gm. WTW (0600 hours).

The GABA levels of the cerebellum also exhibit a unimodal pattern with a peak in the dark phase at 0600 hours. The difference between peak and trough values is significantly different ($P<0.01$). Levels varied from a

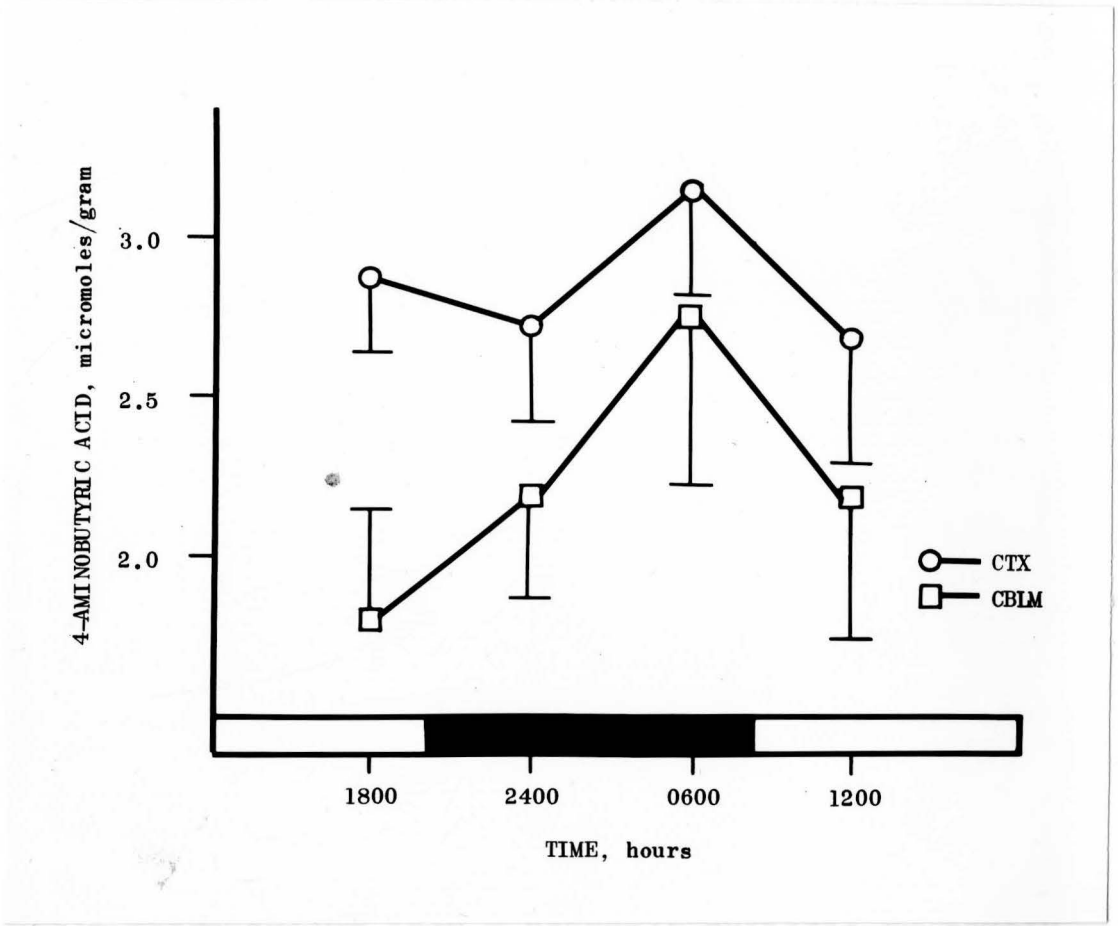


Figure 15. GABA levels in the rat cortex (CTX) and cerebellum (CBLM) over a 24-hour period. The ordinate represents the tissue level of GABA in mcM./gm. WTW. Time is represented on the abscissa. The black area on the abscissa indicates the dark phase of the photoperiod. Vertical brackets indicate the standard deviation (n = 6 animals per point).

trough of 1.80 ± 0.38 mcM./gm. WTW (1800 hours) to a peak value of 2.76 ± 0.53 mcM./gm. WTW (0600 hours).

GABA patterns in the corpora quadrigemina and in the thalamus-hypothalamus are displayed in Figure 16. GABA levels of the corpora quadrigemina exhibit a circadian pattern with a peak during the dark phase. Levels rise gradually, peaking late in the dark phase at 0600 hours; and they decrease to a trough value at 1500 hours. The peak value is significantly different ($P<0.01$) from all other values except the 0900 hour point. The trough value was significantly different ($P<0.01$) for all points except the 1800 hour value. Levels ranged from a trough of 4.28 ± 0.51 mcM./gm. WTW (1500 hours) to a peak of 6.66 ± 0.45 mcM./gm. WTW (0600 hours).

GABA levels of the thalamus-hypothalamus exhibited a multi-modal rhythm with a definite increase in concentrations during the dark phase. The saw-tooth pattern of this rhythm is distinctive, and a statistically significant difference ($P<0.05$) exists between either the 2400 hour point or the 0600 hour point and the 0300 hour point. The 0600 hour point is significantly different ($P< 0.05$) from all other points except the 2400 hour value. Levels ranged from a trough value of 3.82 ± 0.61 mcM./gm. WTW (2100 hours) to a peak value of 5.48 ± 0.29 mcM./gm. WTW (0600 hours).

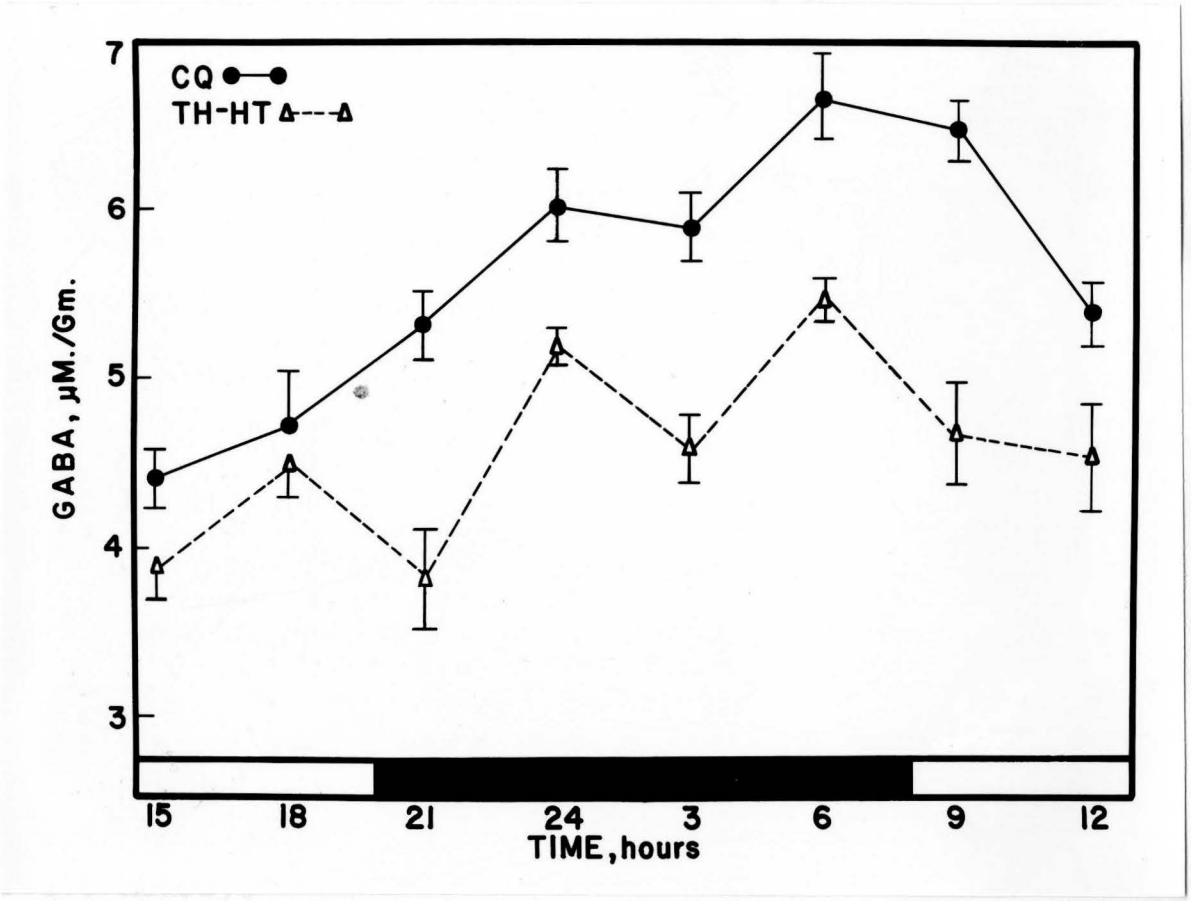


Figure 16. GABA levels in the rat corpora quadrigemina (CQ) and the thalamus-hypothalamus (TH-HT) expressed as a function of time. The ordinate and the abscissa are similar to those of the previous figure. The vertical brackets indicate the standard deviation (n = 6 animals per point).

5. Glycine

The glycine patterns in the rat hindbrain are indicated in Figure 17. In the pons, the glycine levels rise to a peak late in the dark phase, then decrease to a trough in the early light phase. The peak value is significantly different ($P<0.001$) from both of the values obtained in the light phase. It is also significantly different ($P<0.01$) from the other point measured in the dark phase. The levels ranged from a trough value of 2.04 ± 0.37 mcM./gm. WTW (1200 hours) to a peak of 3.56 ± 0.23 mcM./gm. WTW (0600 hours).

Glycine levels of the medulla do not fluctuate as markedly as those of the pons. Levels of this region do not exhibit any significant difference when various time points are compared. The levels ranged from 2.91 ± 0.56 mcM./gm. WTW (1200 hours) to 3.45 ± 0.28 mcM./gm. WTW (1800 hours).

Glycine level patterns in the spinal cord regions are depicted in Figure 18. The patterns of the cervical and lumbar enlargements are similar to each other, but markedly different from the pattern observed in the thoracic cord. The thoracic cord pattern resembles that seen in the pons, with a sharp peak at 0600 hours followed by a rapid fall in levels to a trough at 1200 hours. The peak

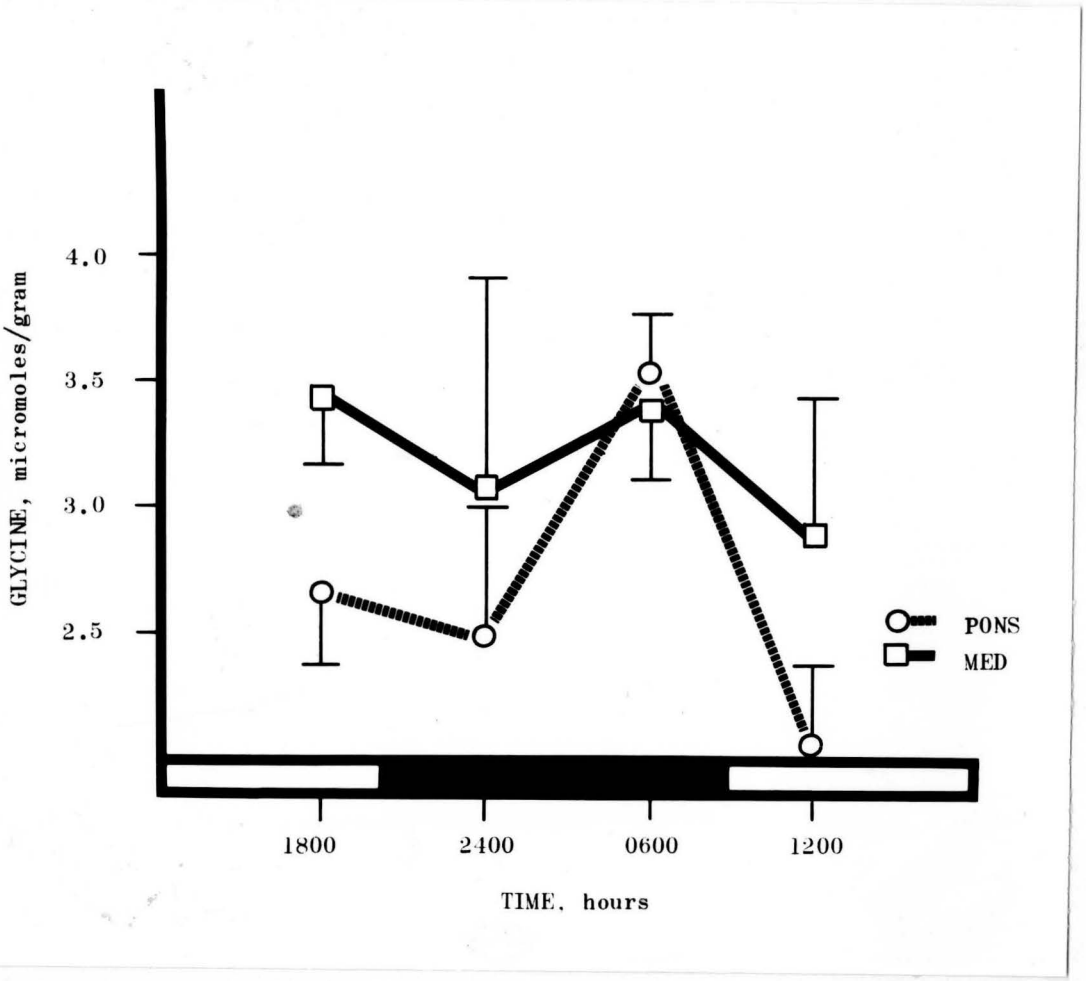


Figure 17. Glycine levels in the rat pons and medulla (MED) expressed as a function of time. The ordinate depicts the tissue levels of glycine in mcM./gm. WTW. Time is noted on the abscissa. The black bar on the abscissa indicates the dark phase of the photoperiod. Vertical brackets indicate the standard deviation (n = 6 animals per point).

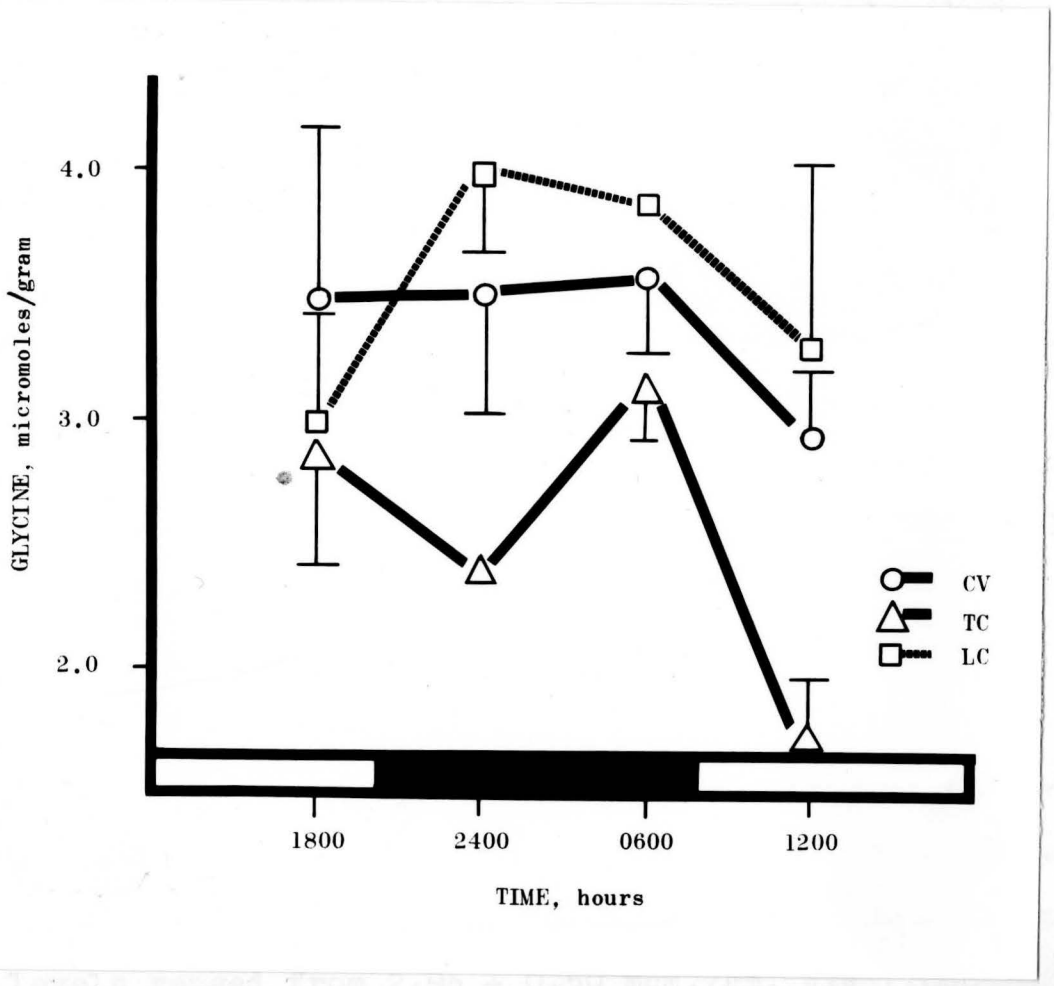


Figure 18. Glycine levels in the rat cervical cord (CV), thoracic cord (TC), and lumbar cord (LC) expressed as a function of time. The ordinate and abscissa are as presented in the previous figure. The vertical brackets indicate the standard deviation (n = 6 animals per point).

and trough values in the thoracic cord are significantly different ($P<0.001$) from each other. Glycine levels ranged from 1.77 $\pm$ 0.21 mcM./gm. WTW (1200 hours) to 3.44 $\pm$ 0.23 mcM./gm. WTW (0600 hours).

Glycine levels in the cervical enlargement exhibit a circadian pattern with a peak at the end of the dark phase. The peak is significantly different ($P<0.01$) from the trough value. However, levels between the points at 0600, 1800, and 2400 hours are not significantly different. Concentrations ranged from 2.96 $\pm$ 0.23 mcM./gm. WTW (1200 hours) to 3.61 $\pm$ 0.30 mcM./gm. WTW (0600 hours).

The glycine pattern in the lumbar cord is similar to that of the cervical cord, except that the peak value occurs at 2400 hours. The peak is not significantly different from the level obtained at 0600 hours. However, it is significantly different ($P<0.01$) from the trough value. The levels ranged from 2.96 $\pm$ 0.50 mcM./gm. WTW (1800 hours) to 4.00 $\pm$ 0.30 mcM./gm. WTW (2400 hours).

The cumulative glycine levels in either the dark or light phase for the tissues above are indicated in Table 1. The medullary glycine levels during light and dark phases are for all practical purposes equivalent. Although glycine levels in both cervical and thoracic cord are higher in the dark phase than those of the light phase, these levels are not significantly different from one another.

	LIGHT PHASE	DARK PHASE
PONS	2.36 ± 0.47 *	3.03 ± 0.67
MEDULLA	3.18 ± 0.51 n.s.	3.26 ± 0.69
CERVICAL CORD	3.23 ± 0.55 n.s.	3.57 ± 0.38
THORACIC CORD	2.32 ± 0.68 n.s.	2.78 ± 0.81
LUMBAR CORD	3.12 ± 0.64 **	3.94 ± 0.10

Table 1. Cumulative glycine levels in the rat pons, medulla, cervical cord, thoracic, and lumbar cord in mcM./gm. WTW ("normal" cycle). Each phase consists of 12 hours. Levels are expressed in micromoles/gram ± standard deviation (n = 12 animals per entry).

* $P < 0.05$ between light and dark phases

** $P < 0.01$ between light and dark phases

n.s. Difference between light and dark phases, not statistically significant.

Significant differences between dark and light phase levels of glycine were obtained in the pons ($P < 0.05$) and the lumbar cord ($P < 0.01$). Table 1 denotes the rostro-caudal gradient differences in glycine concentration described by Aprison and co-workers (1968; 1969b). The levels of glycine are generally higher in the medulla than in the pons, and levels in the thoracic cord are markedly lower than those of the cervical or lumbar enlargements.

C. Effects of Photoperiod Reversal on the Levels of Putative Transmitters

1. Dopamine

DM levels of the rat corpus striatum of animals on a "reverse" cycle display a circadian periodicity (Figure 19). Levels rose throughout the dark phase to a peak at 1800 hours and a trough at 0300 hours. The peak is significantly different ($P < 0.001$) from the trough value. Levels of DM ranged from 6.79 $\pm$ 1.34 mcg./gm. WTW (0300 hours) to 11.12 $\pm$ 0.47 mcg./gm. WTW (1800 hours) and were considerably higher than those of the normal cycle. A significant difference ($P < 0.001$) was obtained between comparable levels of the two cycles. Since the "normal" cycle study was performed in mid-summer, the possibility of a seasonal rhythm must be considered in accounting for

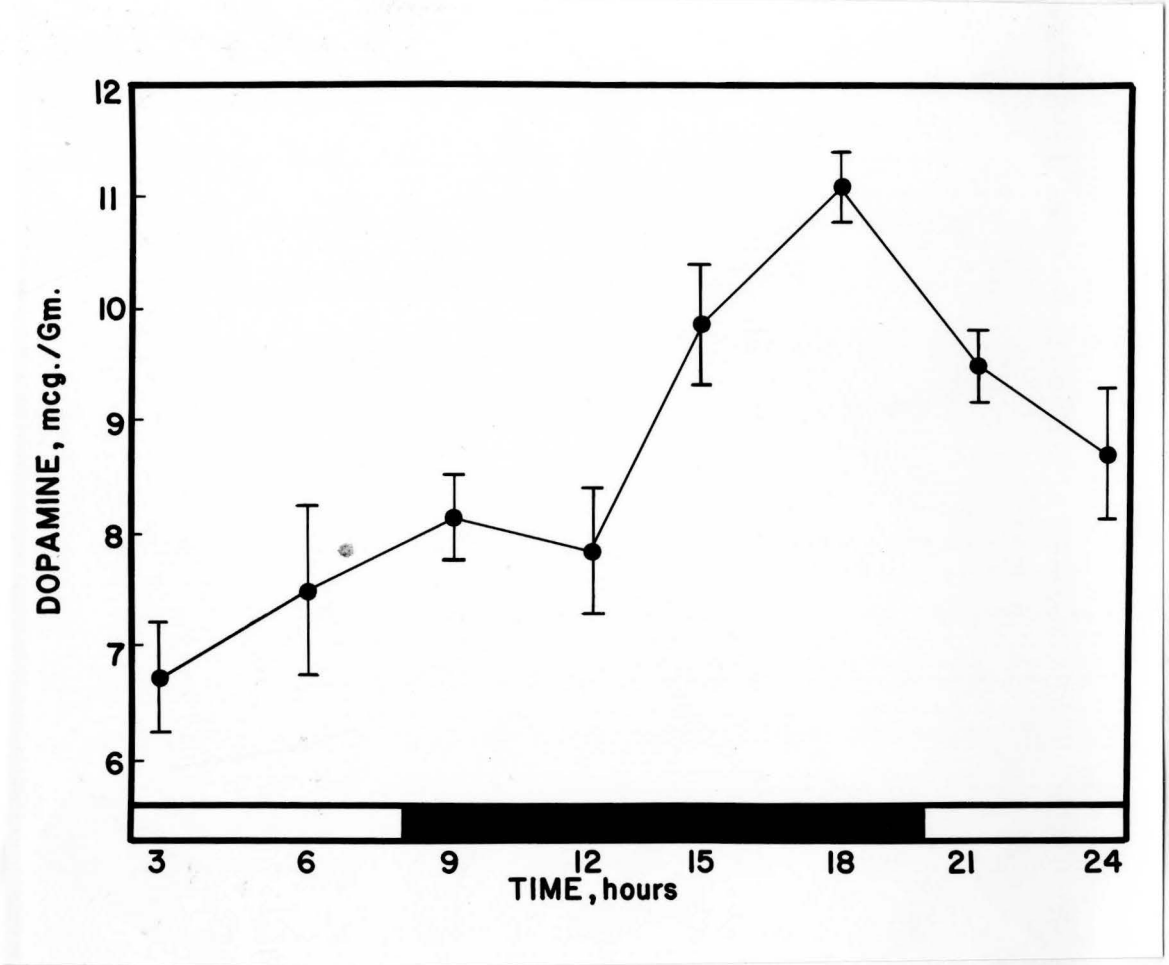


Figure 19. DM levels of the rat corpus striatum as a function of time ("reverse" cycle). The ordinate represents the level of DM in mcg./gm. WTW. Time is represented on the abscissa. The black bar on the abscissa indicates the dark period. Vertical brackets indicate the standard error of the mean (n = 6 animals per point).

this difference in levels.

In an attempt to exclude the seasonal factor, the study was repeated and DM levels were determined simultaneously in animals on the "normal" and "reverse" cycles (this combined study was performed in June, 1970, cf., Figures 12 and 20). The study was extended to include DM levels of the upper and lower brainstem as well as those of the corpus striatum.

The DM levels of the corpus striatum describe a unimodal circadian pattern with a peak at 1200 hours in the dark phase. This point is significantly different ($P < 0.001$) from either point obtained in the light phase. The levels ranged from 5.59 $\pm$ 0.55 mcg./gm. WTW (0600 hours) to 8.74 $\pm$ 0.55 mcg./gm. WTW (1200 hours). This rhythm appeared to be exogenous since it was reversed by photoperiod inversion.

The DM rhythms in both areas of the brainstem were distinctly different from that of the corpus striatum. Brainstem levels decreased during the dark phase of the photoperiod; these rhythms, apparently are endogenous, as the patterns were not reversed by photoperiod inversion. These rhythms are similar to those of Figure 12 when compared on the basis of time and not on the basis of photoperiod interval.

In the case of the upper brainstem, the trough is

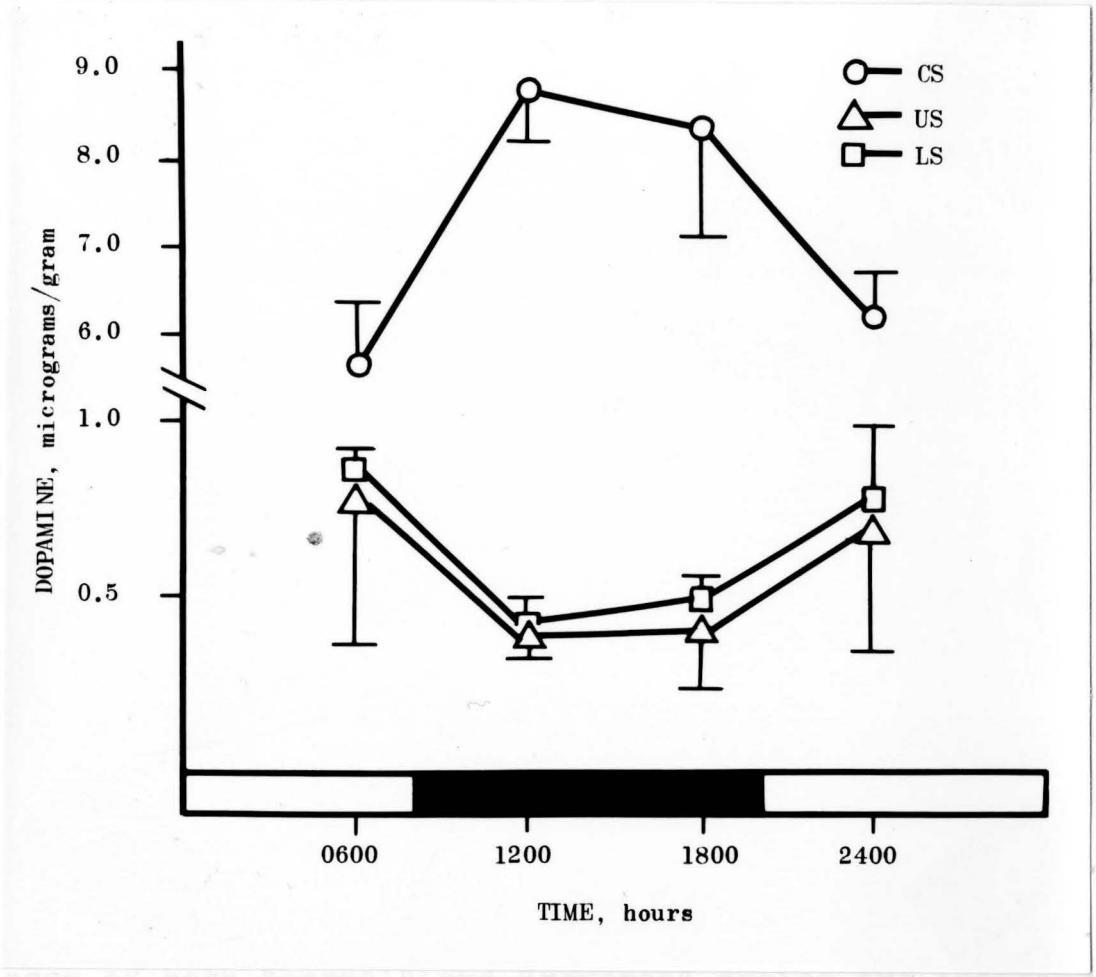


Figure 20. DM levels in the rat corpus striatum (CS), upper brainstem (US), and lower brainstem (LS) expressed as a function of time ("reverse" cycle). The ordinate represents the tissue level of dopamine in mcg./gm. WTW. Time is noted on the abscissa. The black bar on the abscissa indicates the dark phase of the photoperiod. The vertical brackets indicate the standard deviation (n = 6 animals per point).

significantly different ($P < 0.01$) from either point obtained in the light phase. DM levels of the upper brainstem ranged from 0.42 ± 0.08 mcg./gm. WTW (1200 hours) to 0.93 ± 0.23 mcg./gm. WTW (0600 hours).

The trough in DM concentrations of the lower brainstem is highly significant statistically ($P < 0.001$), when compared with the points obtained during the light phase. DM levels in this region ranged from 0.42 ± 0.08 mcg./gm. WTW (1200 hours) to 0.82 ± 0.10 mcg./gm. WTW (0600 hours). In summary, DM levels of the corpus striatum apparently are exogenously controlled since they follow alterations in photoperiod, while those of the upper and lower brainstem apparently are endogenously controlled, since they resist a change due to reversal of photoperiod.

The cumulative levels of DM during the respective phases of both "normal" and "reverse" cycles are indicated in Table 2. In the corpus striatum (column 1), the DM levels are elevated significantly ($P < 0.05$) in the dark phase of the "reverse" cycle over those of the dark phase of the "normal" cycle. In a similar comparison of the light phase levels significance was not proven ($P < 0.10$). As can be seen in Table 2, levels in the dark phase of either cycle are significantly different when compared with those of the light phase.

A comparison of DM levels of the upper brainstem

CYCLE	PHASE	CS	US	LS
NORMAL	LIGHT	5.27 ± 0.88 ***	0.52 ± 0.17 *	0.27 ± 0.13 ***
	DARK	7.19 ± 1.35	0.93 ± 0.45	0.55 ± 0.23
REVERSE	LIGHT	5.90 ± 0.65 ***	0.84 ± 0.21 ***	0.82 ± 0.08 ***
	DARK	8.56 ± 1.01	0.47 ± 0.14	0.43 ± 0.09

Table 2. Cumulative dopamine levels in corpus striatum (CS), upper brainstem (US), and lower brainstem (LS). Levels are expressed in micrograms/gram ± standard deviation (n = 12 animals per point).

* One asterisk indicates a P-value of less than 5%.

*** Three asterisks indicate a P-value of less than 0.1%.

(column 2) in the light and dark phases of either "normal" or "reverse" cycles indicates that they are significantly different from each other. The failure of this rhythm to exhibit reversal is detected by the higher levels found in the light phase of the "reverse" cycle. In other words, relative to clock time levels are not significantly changed. Comparison of the total levels of DM in the upper brainstem of animals on the "normal" cycle versus those on the "reverse" cycle indicates that they are not significantly different.

Comparison of DM levels of light versus dark phase of either cycle in the lower brainstem indicates a highly significant difference (Table 2). However, in this case, DM levels are also significantly higher when the same phases of the "reverse" cycle are compared with those of the "normal" cycle.

In summary, after photoperiod reversal increased levels of DM are found in the corpus striatum and lower brainstem, but not in the upper brainstem.

2. Norepinephrine

NE patterns obtained in the various brain areas studied for animals on a "reverse" cycle are depicted in Figure 21. Peak and trough NE levels of the corpus striatum are not significantly different from each other

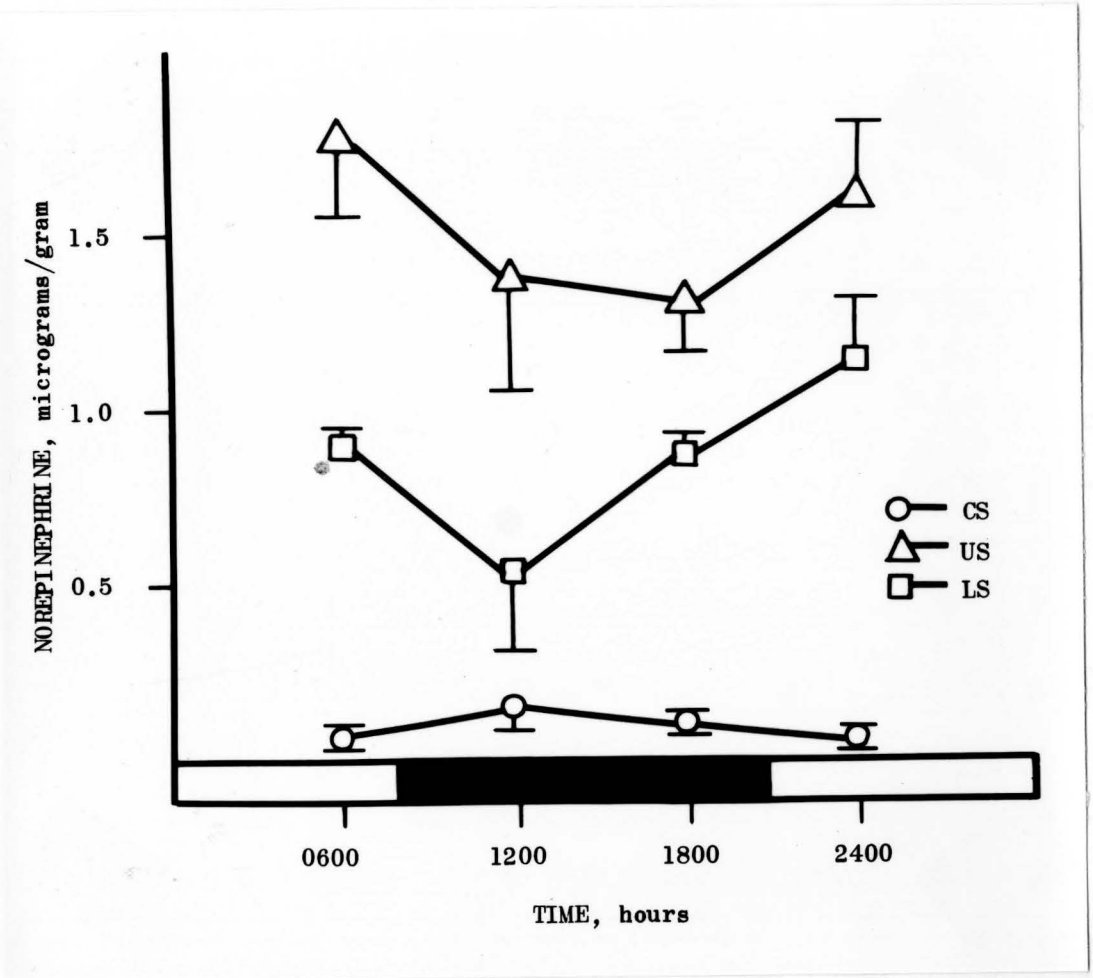


Figure 21. NE levels in the rat corpus striatum (CS), upper brainstem (US), and lower brainstem (LS) expressed as a function of the time of day (reverse cycle). The ordinate represents NE content, mcg./gm. WTW. Time and phases are as in previous figures. Vertical brackets indicate the standard deviation (n = 6 animals per point).

($P < 0.10$). Levels ranged from 0.08 ± 0.03 mcg./gm. WTW (0600 hours) to 0.15 ± 0.08 mcg./gm. WTW (1200 hours). Although only borderline significance was attained, the NE levels of this area exhibit a pattern similar to that of DM on the "reverse" cycle.

The NE patterns in the upper and lower brainstem are in sharp contrast to those obtained in the corpus striatum. NE levels of both brainstem areas trough in the dark phase. In the upper brainstem, the minimal point is significantly different ($P < 0.05$) from those of the light phase. Levels of NE in this area ranged from 1.31 ± 0.14 mcg./gm. WTW (1800 hours) to 1.80 ± 0.19 mcg./gm. WTW (0600 hours).

NE levels of the lower brainstem exhibit a significant ($P < 0.01$) trough during the dark phase. NE levels ranged from 0.54 ± 0.24 mcg./gm. WTW (1200 hours) to 1.15 ± 0.17 mcg./gm. WTW (2400 hours). These brainstem rhythms appear to be unaffected by inversion of the photoperiod and appear to be free-running, or endogenous rhythms.

Cumulative NE levels in these tissues are presented in Table 3. The light/dark phase comparisons of both cycles in all areas studied are significantly different except for levels in the corpus striatum on the "reverse" cycle. NE levels in the corpus striatum during the dark phase of the

CYCLE	PHASE	CS	US	LS
NORMAL	LIGHT	0.07 ± 0.03 ***	1.05 ± 0.22 ***	0.71 ± 0.10 *
	DARK	0.24 ± 0.07	1.74 ± 0.27	0.83 ± 0.11
REVERSE	LIGHT	0.08 ± 0.06 n.s.	1.70 ± 0.25 ***	1.04 ± 0.17 ***
	DARK	0.13 ± 0.07	1.35 ± 0.24	0.71 ± 0.24

Table 3. Cumulative NE levels in rat corpus striatum (CS), upper brainstem (US), and lower brainstem (LS). Each phase consists of 12 hours. Levels are expressed in micrograms/gram ± standard deviation (n = 12 animals per entry).

* One asterisk indicates a P-value of less than 5%.

*** Three asterisks indicate a P-value of less than 0.1%.

n.s. Non-significant difference

"normal" cycle are significantly higher ($P < 0.001$) than the same phase of the "reverse" cycle.

The endogenous characteristics of the NE rhythms in the upper and lower brainstem are evident when one inspects the high NE levels in these areas during the light phase of the "reverse" cycle. Comparison of NE levels in the upper brainstem in the "normal" light phase versus the "reverse" dark phase indicates a significant difference ($P < 0.01$), although there is no difference between the maximal levels of the upper brainstem on these cycles. A significant difference ($P < 0.01$) also exists between the NE levels in the lower brainstem during the "normal" dark as compared with those of the "reverse" light phase.

In summary, the NE rhythms resemble those of DM in these brain regions. As in the case of DM, NE levels seem to be controlled exogenously in the corpus striatum, but endogenously in the brainstem.

3. Serotonin

The 5-HT patterns obtained after photoperiod reversal are depicted in Figure 22. 5-HT levels of the corpus striatum exhibit a circadian pattern with a trough during the dark phase. Thereafter, the levels rise and reach a peak late in the light phase. 5-HT levels ranged from a trough value of 0.54 ± 0.17 mcg./gm. WTW

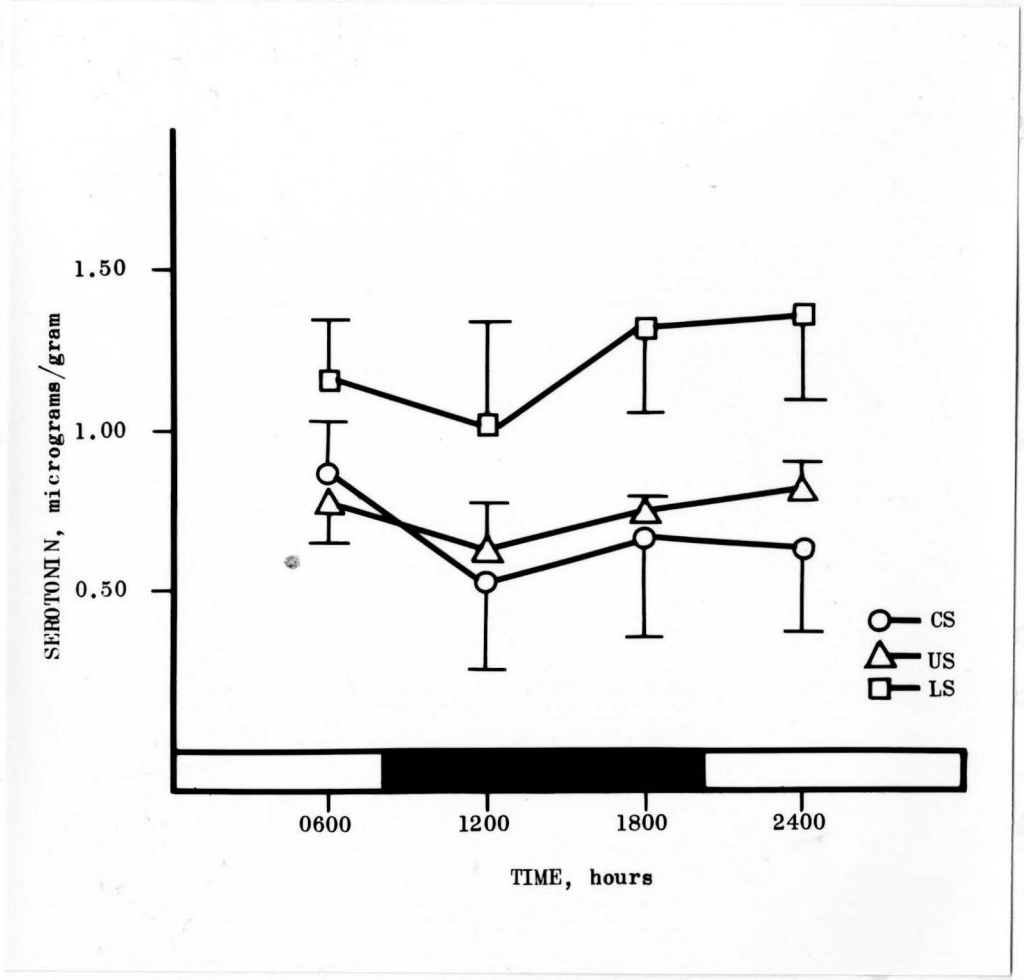


Figure 22. 5-HT levels in the rat corpus striatum (CS), upper brainstem (US), and lower brainstem (LS) expressed as a function of time of day (reverse cycle). The ordinate represents 5-HT content in mcg./gm. WTW. Time and phases are as in previous figures. Vertical brackets indicate the standard deviation (n = 6 animals per point).

(1200 hours) to a peak value of 0.86 ± 0.17 mcg./gm. (0600 hours). These two values are significantly different at the 5% significance level.

5-HT levels in the upper brainstem also exhibit a unimodal circadian pattern with a trough in the dark phase. Both values of the dark phase are significantly different ($P < 0.05$) from the peak value. Levels ranged from 0.62 ± 0.07 mcg./gm. WTW (1200 hours) to 0.82 ± 0.07 mcg./gm. WTW (2400 hours).

5-HT levels in the lower brainstem do not vary significantly over 24 hours. Photoperiod reversal apparently counteracts the rhythm obtained on the "normal" cycle. The levels in this area ranged from 1.04 ± 0.35 mcg./gm. WTW (1200 hours) to 1.41 ± 0.31 mcg./gm. WTW (2400 hours).

Cumulative 5-HT levels are compared in Table 4. Statistically significant dark/light differences were found for all comparisons of 5-HT on the "normal" cycle, but only for the upper brainstem on the "reverse" cycle. Levels of 5-HT are generally higher in the light phase except in the lower brainstem on the "normal" cycle. When similar phases of different cycles are compared, the only significant difference ($P < 0.01$) found occurs in a comparison of the levels of the "normal" light phase to those of the "reverse" dark phase in the lower brainstem. Reversal of the illumination

CYCLE	PHASE	CS		US		LS	
NORMAL	LIGHT	0.81 ± 0.29	***	0.85 ± 0.16	*	0.84 ± 0.21	*
	DARK	0.49 ± 0.15		0.67 ± 0.13		1.10 ± 0.29	
REVERSE	LIGHT	0.76 ± 0.24	n.s.	0.80 ± 0.09	***	1.28 ± 0.28	n.s.
	DARK	0.60 ± 0.28		0.67 ± 0.11		1.18 ± 0.31	

Table 4. Cumulative serotonin levels in corpus striatum (CS), upper brainstem (US), and lower brainstem (LS). Levels are expressed in micrograms/gram ± standard deviation. Each phase consists of 12 hours light or 12 hours dark.

* One asterisk indicates a P-value of less than 5%

*** Three asterisks indicate a P-value of less than 0.1%

n.s. Non-significant difference

schedule apparently affects 5-HT levels in a different manner than it does catecholamine levels.

4. 4-aminobutyric Acid (GABA)

The effects of photoperiod inversion on GABA levels are shown in Figure 23 and 24.

The GABA rhythm of the frontal cortex (Figure 23) shows a trough in the light phase with a gradual rise to a peak late in the dark phase. In contrast to the rhythm of cortical GABA levels on the "normal" cycle, the reversed rhythm is highly significant ($P < 0.01$) when peak and trough values are compared. The levels ranged from a trough value of 2.24 ± 0.35 mcM./gm. WTW (0600 hours) to a peak value of 3.00 ± 0.23 mcM./gm. WTW (1800 hours).

GABA levels of the cerebellum in animals on the reverse cycle exhibit a rhythm similar to that of cortical GABA i.e., a unimodal pattern with a peak late in the dark phase. A significant difference ($P < 0.05$) between the peak and trough values was obtained. The range of GABA concentrations varied from 1.90 ± 0.24 mcM./gm. WTW (2400 hours) to a peak of 2.35 ± 0.34 mcM./gm. WTW (1800 hours).

On the reversed cycle, GABA levels of the corpora quadrigemina (Figure 24) exhibit an unusual circadian pattern. Both the peak and trough occurred during the dark phase. It suggests the possibility that the rhythm of the

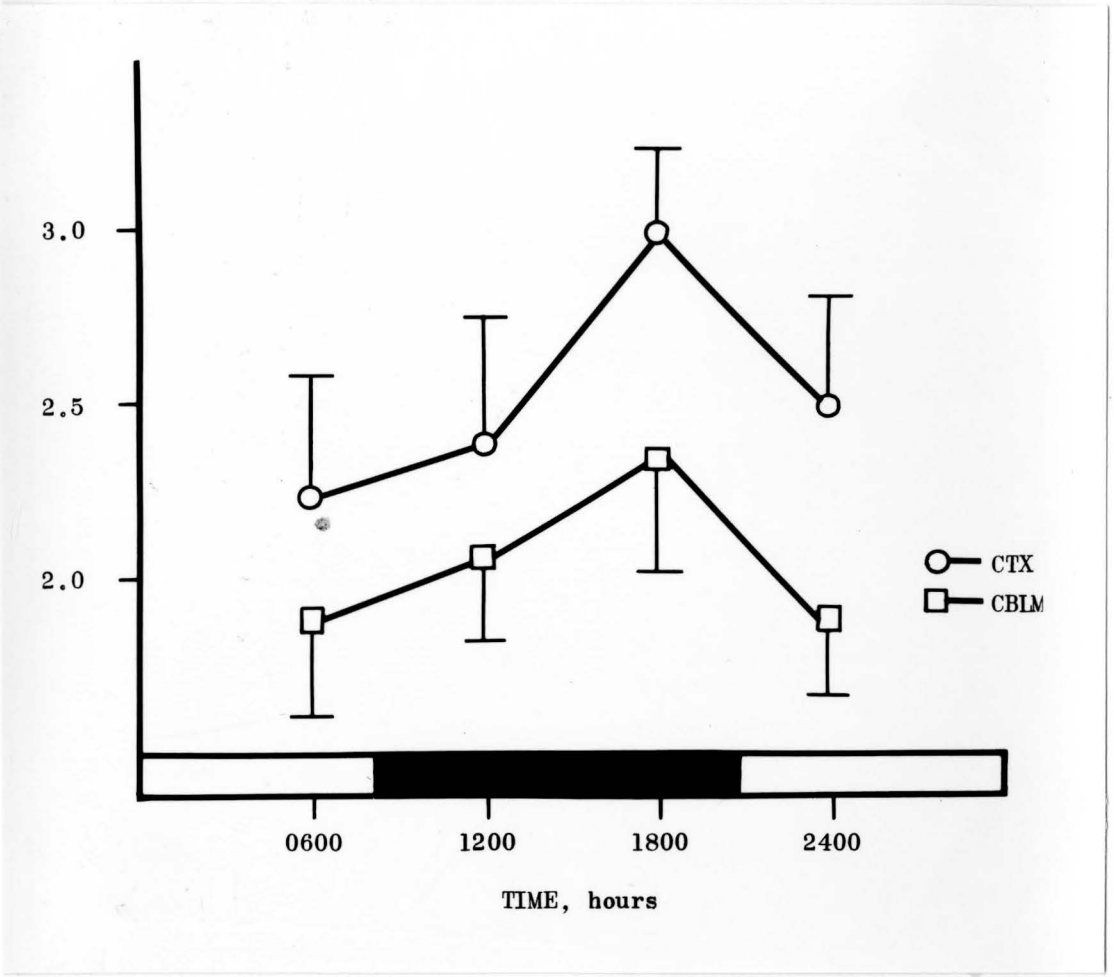


Figure 23. The levels of GABA in the frontal cortex (CTX) and cerebellum (CBLM) expressed as a function of the time of day ("reverse" cycle). The ordinate represents the tissue levels of GABA in mcM./gm. WTW. Time is noted in the abscissa. The dark bar on the abscissa indicates the dark phase of the photoperiod. Vertical brackets indicate the standard deviation (n = 6 animals per point).

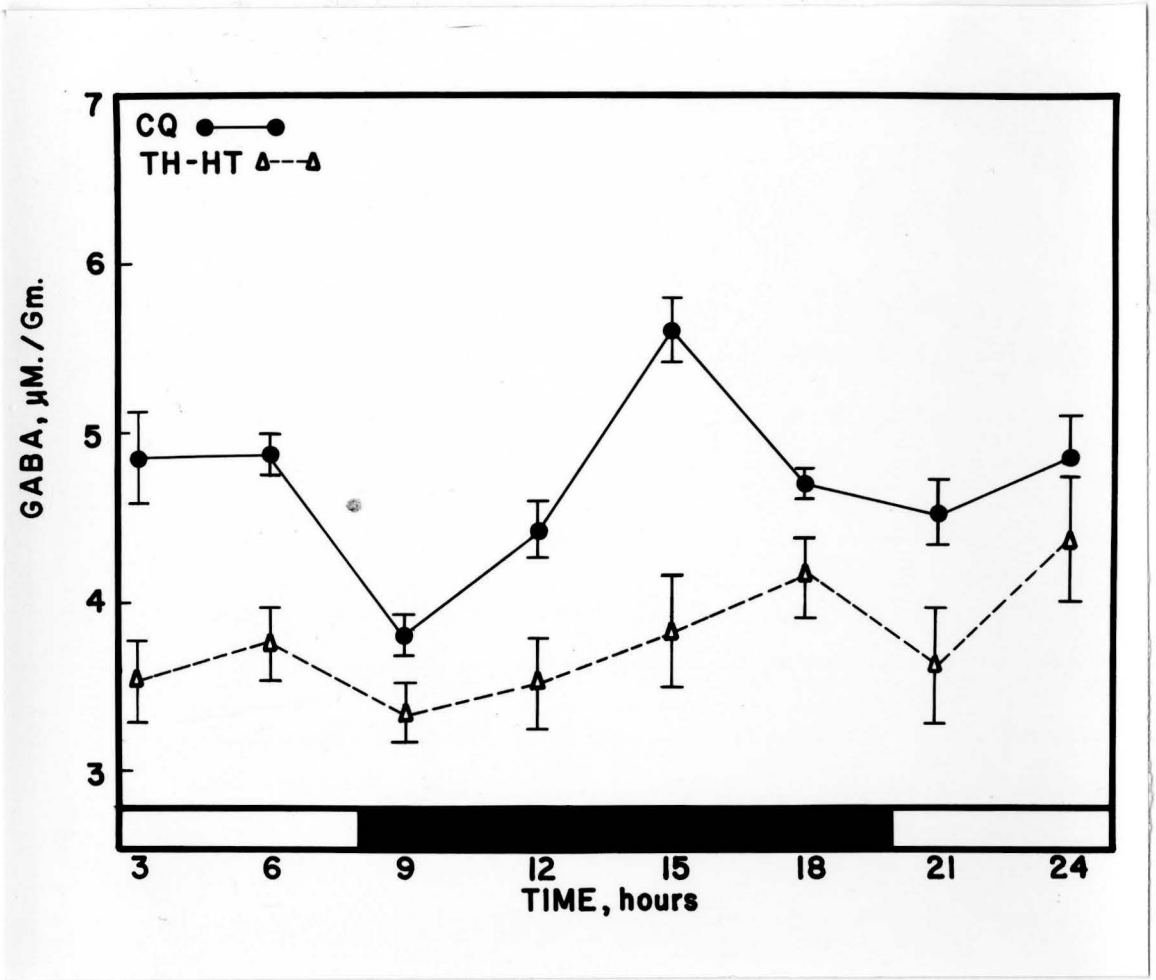


Figure 24. The levels of GABA in the corpora quadrigemina (CQ) and the thalamus-hypothalamus (TH-HT) expressed as a function of time of day ("reverse" cycle). The ordinate, abscissa, and vertical brackets are as in the previous figure.

corpora quadrigemina resists reversal, and that if the period of adaptation were increased, a more defined reversal rhythm would be obtained. GABA levels throughout the light phase seem remarkably stable. They fall at the onset of the dark phase, and rise sharply to a peak at 1500 hours. The trough value at 0900 hours is significantly different ($P < 0.05$) from all other points in the dark phase. Levels varied from 3.77 ± 0.33 mcM./gm. WTW (0900 hours) to 5.66 ± 0.62 mcM./gm. WTW (1500 hours).

The GABA pattern for the thalamus-hypothalamus is also different from previous rhythms shown in Figure 24. As on the "normal" cycle, the levels fluctuate in a saw-tooth fashion (Figure 25). This rhythm possibly is an endogenous six hour ultradian rhythm superimposed on an exogenous circadian pattern. There is significant difference ($P < 0.01$) between peak and trough values in this case. The levels ranged from 3.30 ± 0.27 mcM./gm. WTW (0900 hours) to 4.11 ± 0.67 mcM./gm. WTW (2400 hours).

Cumulative GABA levels are compared in Table 5. The light/dark phase comparisons for each tissue region are presented. The only region exhibiting a significant, readily reversible rhythm, was the cerebellum. Comparison of "normal" and "reverse" cycle levels in the cerebellum indicates no significant differences. Levels of cortical

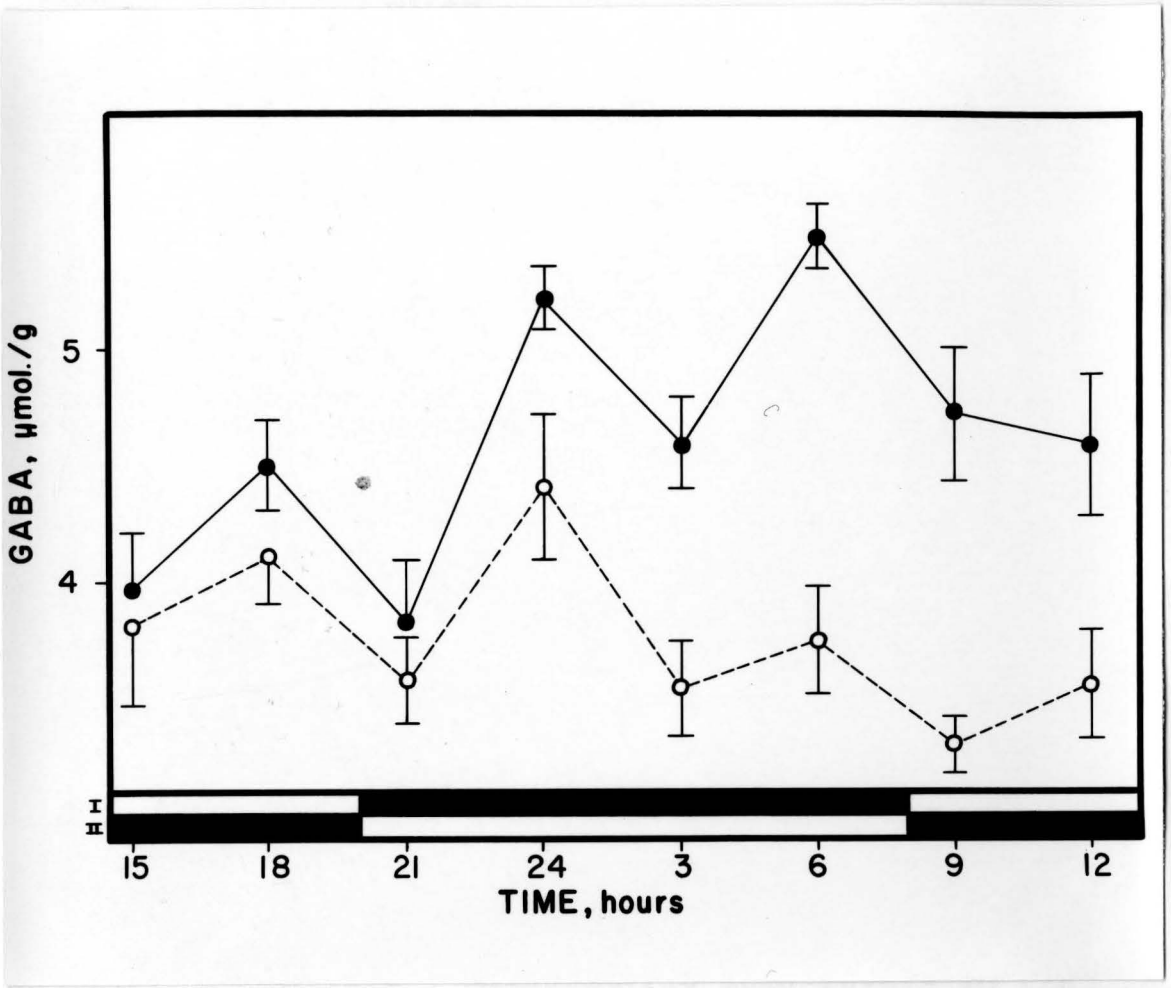


Figure 25. The levels of GABA in the thalamus-hypothalamus on both the "normal" (I) and "reverse" (II) cycles which are indicated on the abscissa. The ordinate and vertical brackets are as in the previous figure. Closed circles and solid lines represent the normal cycle rhythm, and open circles and dotted lines the reverse cycle rhythm.

NORMAL CYCLE

	LIGHT PHASE		DARK PHASE
CQ	5.24 ± 0.49	***	5.89 ± 0.36
TH-HT	4.45 ± 0.34	**	4.78 ± 0.39
CTX	2.78 ± 0.29	n.s.	2.94 ± 0.42
CBLM	2.00 ± 0.44	*	2.47 ± 0.51

REVERSE CYCLE

	LIGHT PHASE		DARK PHASE
CQ	4.79 ± 0.21	n.s.	4.64 ± 0.41
TH-HT	3.82 ± 0.32	n.s.	3.70 ± 0.32
CTX	2.38 ± 0.34	*	2.71 ± 0.42
CBLM	1.91 ± 0.26	*	2.21 ± 0.30

Table 5. Cumulative 4-aminobutyric acid levels in the corpora quadrigemina (CQ), thalamus-hypothalamus (TH-HT), frontal cortex (CTX), and cerebellum (CBLM). Levels are expressed in micromoles/gram ± standard deviation (n = 12 animals per entry). Each phase consists of 12 hours light or 12 hours dark. Significance is indicated by asterisks as in previous tables. "n.s." indicates non-significant difference.

GABA on the normal cycle are significantly higher ($P < 0.01$) than those of the "reverse" cycle. Comparisons of levels of similar phases of different cycles in the corpora quadrigemina and thalamus-hypothalamus indicate that the normal cycle levels are significantly higher ($P < 0.001$) than those of the "reverse" cycle.

D. Response to MES as a Function of Time: Vehicle Controls

The duration of various components of MES seizures during a normal cycle in control animals are presented in Table 6. Both saline and the suspending agent, 5% acacia, were evaluated in order to determine effects of the vehicles. All mice used in these groups had been carefully selected for a single body weight (34 grams) to facilitate the detection of small changes in the time course of the seizure pattern.

Each animal exhibited a full tonic-clonic type seizure. Two deaths occurred in every group of ten, except the 0600 hour acacia group, in which one death occurred. Animals which died during the seizure were excluded from these statistics. All animals exhibited post-ictal depression and short-term refractoriness (30-45 seconds) to subsequent shock. The onset time of tonic extension (extensor latency) in the saline-injected group was

TIME	TREATMENT	EXTENSOR LATENCY, SECONDS	TONIC EXTENSION, SECONDS	CLONUS, SECONDS
0600	0.9% Saline	1.86 ± 0.28	13.74 ± 1.52	6.10 ± 1.44
1800	0.9% Saline	2.17 ± 0.32	13.29 ± 0.86	7.69 ± 2.83
0600	5% Acacia	2.17 ± 0.36	15.77 ± 1.13	6.79 ± 2.59
1800	5% Acacia	2.12 ± 0.31	14.88 ± 1.41	6.58 ± 2.06

Table 6. Time, in seconds, for seizure components of normal saline and 5% acacia-treated control mice at 0600 and 1800 hours. Values are expressed ± standard deviation (n = 8 for all groups except the 0600 hour acacia group, in which n = 9).

decreased ($P < 0.05$) at 0600 hours when compared with the group at 1800 hours. However, there was no significant difference between these groups in the duration of tonic extension or of clonus. Seizure component times of the acacia-treated groups were not significantly different in a similar comparison.

An evaluation of seizure component times of saline-treated animals versus those of acacia-treated animals at similar times revealed significant differences ($P < 0.05$) in the duration of tonic extension at 0600 and 1800 hours, and the duration of extensor latency at 0600 hours.

The minimal electroshock threshold (Swinyard, et al., 1952) was determined at 0600 and 1800 hours. The minimal seizure pattern is generally of the alternating limb-clonic type, although some mice will exhibit a tonic-clonic pattern at these low currents. The minimal threshold current was 9.67 ± 1.10 ma. at 1800 hours ($n = 9$), and 10.29 ± 0.98 ma. at 0600 hours ($n = 9$); however, these values did not differ significantly from each other. The percent of mice dying at 10 ma. of current was higher at 0600 hours (33%), than at 1800 hours (11%), but this difference was also not statistically significant when tested by the Chi-square method.

E. Circadian Variations in the Efficacy of Drugs

1. Convulsant Drugs

a. Allylglycine

The circadian toxicity (LD50) of two potent central stimulants is graphed in Figure 26. The lethality of allylglycine was significantly higher ($P < 0.05$) in the light (especially at 1800 hours) than in the dark phase. Animals receiving allylglycine exhibited signs of increased motor activity and hyperexcitability that led to convulsive episodes in a majority of them. Animals which convulsed exhibited a typical seizure pattern of clonic movements of the limbs followed by flexion and extension (and sometimes a short clonus). When death occurred, it generally followed repetitive convulsions of the clonic-tonic type, with the animals dying in extension. All deaths occurred within ninety minutes after injection of the compound.

b. Strychnine Sulfate

The pattern for strychnine sulfate toxicity was similar to that of allylglycine. A significant difference ($P < 0.05$) occurred between the LD50's at 0600 and 1800 hours. The toxicity of this compound is maximal during the light phase, and minimal in the dark phase of the illumination cycle. After receiving the drug, all animals exhibited

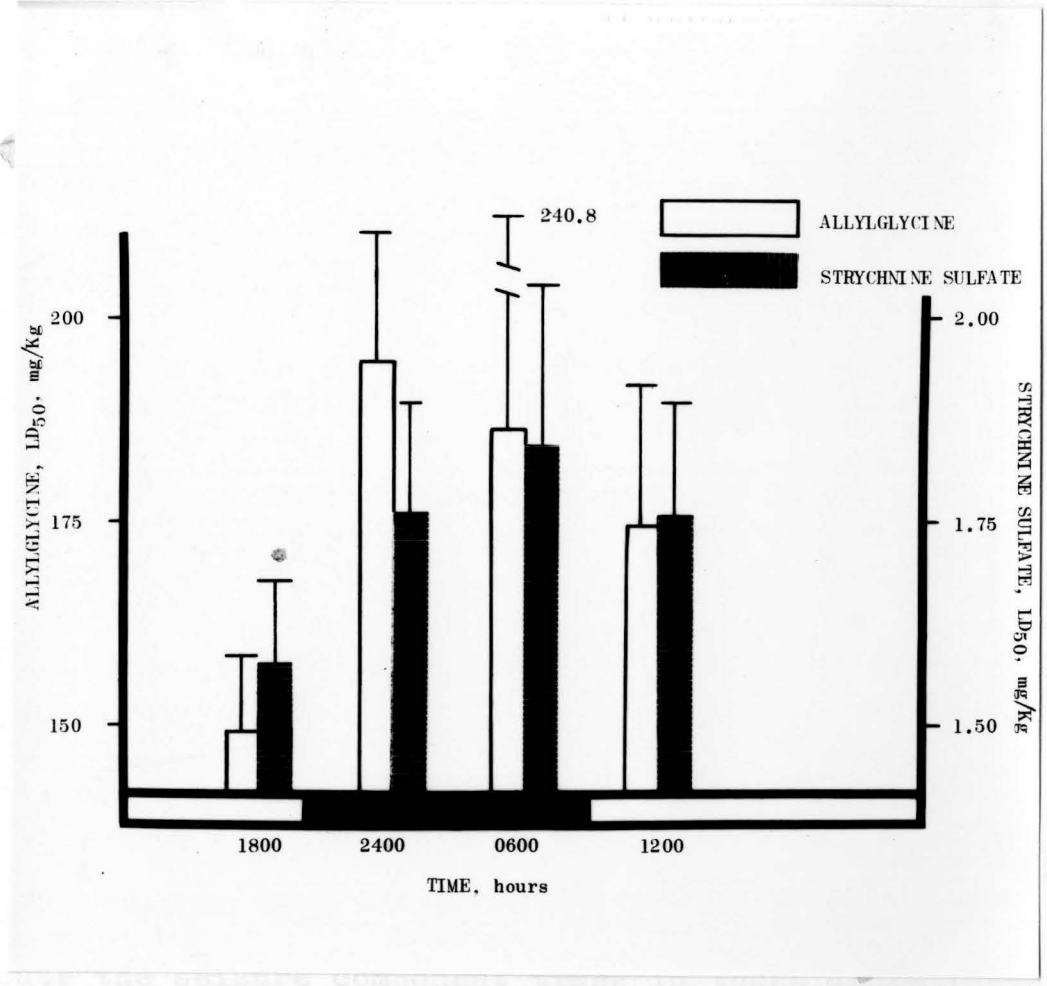


Figure 26. The circadian toxicity of two convulsant drugs, allylglycine and strychnine sulfate. The ordinate depicts the dose of each respective drug in mg./Kg. The abscissa indicates the time of day CST, with the black bar marking the dark phase of the photoperiod. Vertical brackets indicate the standard deviation (n = 18 animals per determination of LD50).

classic signs of increased motor activity, increased responsiveness to auditory stimuli, and hyperexcitability. Animals convulsing displayed opisthotonus with an extreme tonic extension terminating in death. All deaths occurred within ninety minutes after injection of the drug.

2. Anti-convulsant Drugs

a. Acetazolamide

The PD50's with respect to MES for acetazolamide in both light and dark phases are indicated in Tables 7 (mice) and 8 (rats). Animals receiving this drug could not be differentiated from control animals with regard to motor function. No changes in neurological functions were evident. The PD50 of the drug did not vary significantly between the test times. Table 9 (mice) and Table 10 (rats) indicate the seizure component times in those drug-treated animals that exhibited seizures at the low dosage level. After acetazolamide, the extensor latency time was significantly elevated ($P < 0.001$) from that of control animals of both species in both phases. In mice, the duration of clonus was significantly increased ($P < 0.05$) from values in the light phase (1800 hours). Other times of rodent seizure components were not significantly different from control.

DRUG	TIME 0600 (dark)		1800 (light)	
Acetazolamide	84.45 ± 15.35	n.s.	91.00 ± 40.43	
Diphenylhydantoin	9.79 ± 2.18	n.s.	9.26 ± 1.48	
Meprobamate	187.40 ± 8.30	***	152.90 ± 9.70	
Phenobarbital Sodium	23.68 ± 4.40	n.s.	23.04 ± 1.01	

Table 7. The PD50 of anti-convulsant drugs in mice during light or dark phase of the illumination cycle. Values are expressed in mg./Kg., I.P., ± standard deviation (n = 18 mice per determination).

*** = $P < 0.001$

n.s. = non significant difference

DRUG	TIME 0300 (dark)		1500 (light)	
Acetazolamide	9.80 ± 6.30	n.s.	10.88 ± 3.22	
Diphenylhydantoin	8.47 ± 4.39	n.s.	8.47 ± 4.39	
Meprobamate	90.82 ± 26.50	x	76.98 ± 10.12	
Phenobarbital Sodium	5.85 ± 2.86	n.s.	5.85 ± 2.86	

Table 8. The PD50 of anti-convulsant drugs in rats during the dark or light phase of the illumination cycle. Values are expressed in mg./Kg., I.P., ± the standard deviation (n = 14 rats per determination).

x = $P < 0.10$

n.s. = non-significant difference

Table 9. Time, in seconds, for seizure components of normal saline control and drug-treated mice which still showed convulsions. Values are expressed ± standard deviation.

TIME	DRUG TREATMENT	n	EXTENSOR LATENCY	TONIC EXTENSION	CLONUS
0600	Saline, 0.9%	10	1.86 ± 0.28	13.74 ± 1.52	6.10 ± 1.44
1800	Saline, 0.9%	10	2.17 ± 0.32	13.29 ± 0.86	7.69 ± 2.83
0600	Acetazolamide, 80 mg./Kg.	5	3.86 ± 0.61	13.06 ± 3.54	10.70 ± 4.30
1800	Acetazolamide, 80 mg./Kg.	5	3.64 ± 0.49	11.72 ± 3.30	11.94 ± 3.78
0600	Diphenylhydantoin, 8 mg./Kg.	6	4.00 ± 0.55	6.85 ± 2.73	27.87 ±13.03
1800	Diphenylhydantoin, 8 mg./Kg.	6	3.95 ± 0.67	10.95 ± 4.13	16.16 ± 5.58
0600	Meprobamate, 180 mg./Kg.	6	4.57 ± 0.50	7.92 ± 1.16	12.82 ± 5.15
1800	Meprobamate, 150 mg./Kg.	5	3.92 ± 1.36	9.14 ± 3.95	10.72 ± 2.33
0600	Phenobarbital Sodium, 20 mg./Kg.	6	3.87 ± 0.54	7.08 ± 2.38	14.38 ± 3.52
1800	Phenobarbital Sodium, 20 mg./Kg.	8	4.58 ± 1.55	7.63 ± 4.07	18.85 ± 4.43

Table 10. Time, in seconds, for seizure components of normal saline control and those drug-treated rats which still showed convulsions. Values are expressed ± standard deviation.

TIME	DRUG TREATMENT	n	EXTENSOR LATENCY	TONIC EXTENSION	CLONUS
0300	Saline, 0.9%	7	1.98 ± 0.10	10.72 ± 1.62	17.48 ± 7.30
1500	Saline, 0.9%	7	1.95 ± 0.10	11.42 ± 1.03	16.11 ± 5.51
0300	Acetazolamide, 8 mg./Kg.	4	3.87 ± 0.90	8.85 ± 0.80	22.47 ± 11.13
1500	Acetazolamide, 8 mg./Kg.	5	3.12 ± 0.24	9.72 ± 2.21	20.44 ± 10.58
0300	Diphenylhydantoin, 5 mg./Kg.	5	2.82 ± 0.63	10.52 ± 1.59	25.36 ± 10.31
1500	Diphenylhydantoin, 5 mg./Kg.	5	3.28 ± 0.44	7.08 ± 2.31	28.36 ± 9.36
0300	Meprobamate, 67 mg./Kg.	5	3.70 ± 0.55	8.40 ± 1.53	21.74 ± 5.97
1500	Meprobamate, 67 mg./Kg.	5	3.24 ± 0.37	8.46 ± 2.76	26.04 ± 4.23
0300	Phenobarbital Sodium, 5 mg./Kg.	4	3.90 ± 0.67	7.95 ± 2.05	24.10 ± 5.76
1500	Phenobarbital Sodium, 5 mg./Kg.	4	3.77 ± 0.38	7.15 ± 2.23	29.37 ± 8.44

There was no significant difference in seizure component times between acetazolamide-treated rodents in light or dark phases.

b. Diphenylhydantoin

Rodents receiving diphenylhydantoin exhibited normal motor activity and no apparent neurological deficits. The PD50 of diphenylhydantoin in the dark phase did not differ significantly from that of the light phase in either species (Table 7 and 8).

Seizure component times of convulsed animals, are indicated in Tables 9 and 10. Extensor latency and duration of clonus times in both phases were significantly increased ($P < 0.001$) above those found in the control mice, while the duration of tonic extension was decreased significantly ($P < 0.05$). In rats, extensor latency time was increased significantly ($P < 0.001$) from that of control, but the time of clonus was significantly increased ($P < 0.05$) only during the light phase.

A comparison of seizure component times in the diphenylhydantoin-treated rats of different phases indicated that the extension phase was significantly decreased ($P < 0.01$) in the light phase. A similar comparison in mice indicated only borderline significance ($P < 0.10$) in the following parameters: increased duration of tonic

extension in the light phase, and increased duration of clonus in the dark phase. All other comparisons of seizure component times were not significantly different in different phases with either species.

c. Meprobamate

Anti-convulsant doses of meprobamate produced sedation and muscle relaxation in the treated animals. They were unable to maintain their balance on the edge of a cage and did not replace their hindlimb at a level surface as described (cf. Methods section III-D2-c). The PD50 of meprobamate obtained during the dark phase was significantly higher ($P < 0.001$) than that obtained during the light phase in mice (Table 7); somewhat similar data were obtained in the rat, but the difference was only of borderline significance ($P < 0.10$; Table 8).

Comparison of seizure component times of treated rodents versus controls (Tables 9 and 10) revealed the following significant differences: increased extensor latency time ($P < 0.001$), decreased tonic extensor duration ($P < 0.05$) in both species during both phases, and increased duration of clonus ($P < 0.05$) of mice in both phases and of the rat in the light phase only ($P < 0.01$).

Comparison of seizure component times of meprobamate-treated animals in different phases yielded no

significant difference in either species.

d. Phenobarbital Sodium

Phenobarbital sodium in doses greater than 10 mg./Kg. produced sedation with no apparent neurological deficit in the treated animals. The PD50 of phenobarbital sodium did not differ significantly between light and dark phases (Table 7 and 8).

Seizure component times for phenobarbital-treated rodents during light and dark phases are indicated in Tables 9 and 10. Seizure extensor latency and duration of clonus were increased significantly ($P < 0.001$) from control values in mice. The duration of tonic extension in mice was decreased significantly ($P < 0.05$) from control. In rats, extensor latency time was increased significantly ($P < 0.001$) over that of control in both phases. However, time of clonus is significantly increased ($P < 0.05$) and duration of tonic extension significantly decreased ($P < 0.05$) only in the light phase.

Comparison of seizure component times in phenobarbital-treated animals in the light and dark phases indicated no significant differences.

e. Amino-oxyacetic Acid

Amino-oxyacetic acid (AOAA) was described as an anti-convulsant drug by Kuriyama and co-investigators (1966).

Attempts to duplicate their results in our laboratory were fruitless. Preliminary testing with the other anti-convulsant substances consisted of treating groups of five animals each with a series of doses to determine the approximate PD50. Doses of 5, 15, 20, 30, 50, and 100 mg./Kg. of AOAA I.P., were used. No protection was observed with these doses when challenged one-hour later with MES, although the data of the investigators cited indicated that a 52 mg./Kg. dose (25 mg./Kg. of the base) gave approximately 80-90% protection from seizure at that time. Forty percent of the mice died at the 100 mg./Kg. dose level. Seizure component times at these doses are indicated in Table 11. Comparison of these AOAA-treated mice with pooled saline controls (from Table 9) indicates that AOAA decreased extensor latency time significantly by doses of 15, 30 (P 0.05), 50, and 100 mg./Kg. ($P < 0.001$). Duration of clonus was increased significantly ($P < 0.05$) at the 50 mg./Kg. dose.

To reproduce the original study of Kuriyama and co-investigators (1966), 12 mice were fasted for 18 hours prior to the administration of 50 mg./Kg. of AOAA hemihydrochloride. Groups of three mice were then shocked at ½, 1, 1½, and 2 hours following the injection of drug (Table 12). A significant increase ($P < 0.05$) in the

DOSE mg./Kg.	EXTENSOR LATENCY seconds	TONIC EXTENSION seconds	CLONUS seconds
5	2.30 ± 0.66	13.30 ± 1.45	19.48 ± 18.19
[illegible]	1.68 ± 0.13*	17.32 ± 3.30*	5.66 ± 4.17
20	2.22 ± 0.15	15.18 ± 1.98	8.48 ± 2.74
30	2.44 ± 0.23*	15.40 ± 0.97*	7.40 ± 1.80
50	2.46 ± 0.86	21.15 ± 2.40***	10.55 ± 2.90*
100	2.53 ± 0.58*	24.03 ± 6.39***	8.47 ± 1.42

Table 11. The effects of I.P. doses of AOAA on various component times of MES seizures in mice. Drugs were administered at 1200-1400 hours. Testing was begun at 1400 hours, and concluded at 1600 hours. Values are shown in seconds ± their respective standard deviation (n = 5). Significance against pooled saline controls from 0600 and 1800 hours is indicated by:

* $P<0.05$

*** $P<0.001$

post drug, min.	EXTENSOR LATENCY seconds	TONIC EXTENSION seconds	CLONUS seconds
CONTROL	2.02 ± 0.27	13.57 ± 1.19	6.90 ± 2.10
30	1.97 ± 0.10	17.90 ± 4.40	6.15 ± 0.85
60	2.70 ± 0.75	15.90 ± 1.65*	8.10 ± 1.90
90	2.15 ± 0.35	16.22 ± 2.00*	8.10 ± 1.55
120	2.43 ± 0.25	19.23 ± 1.65***	6.44 ± 1.55
AOAA average (n = 12)	2.31 ± 0.36	17.31 ± 2.42***	7.19 ± 1.46

Table 12. The effects of AOAA, 50 mg./Kg., I.P., on MES seizure component times at various times after administration in mice. Values are expressed in seconds ± standard deviation (n = 3). Averaged saline controls are included for comparative purposes.

* = $P < 0.05$

*** = $P < 0.001$

duration of tonic extension was noted at 60, 90, and 120 minutes after the administration of AOAA. If all of the times of seizure phases are accumulated and evaluated, a significant increase ($P < 0.001$) is also seen in this parameter.

To assure that the ineffectiveness of AOAA to produce any anti-convulsant action was not related to the time of day, animals were fasted for eighteen hours and tested at 0600 and 1800 hours with a MES challenge 1.5 hours after the administration of either saline or AOAA, 50 or 70 mg./Kg., I.P. (Table 13). The current was lowered to 15 ma. because Kuriyama et al. (1966) used a current approximately 25% over threshold current in their strain of mice. Seizure patterns after 50 mg./Kg. dose were not significantly different from the saline control except for an increased extensor latency at 0600 hours ($P < 0.05$). However, the 70 mg./Kg. dose level induced significant increases in both the extensor latency ($P < 0.05$) and the duration of tonic extension ($P < 0.01$) at 0600 and 1800 hours.

In summary, AOAA was ineffective as an anti-convulsant compound, but is capable of increasing extensor latency and prolonging the duration of tonic extension in the Ha/ICR strain of mice.

Table 13. Seizure component times, in mice, during both light (1800 hours) and dark (0600 hours) phases of the illumination cycle following AOAA hemi-hydrochloride administration, 50 or 70 mg./Kg. Values are expressed in seconds ± standard deviation.

* = P 0.05 ** = P 0.01 *** = P 0.001

TIME	DRUG TREATMENT	n	EXTENSOR LATENCY seconds	TONIC EXTENSION seconds	CLONUS seconds
0600	0.9% Saline, I.P.	7	2.24 ± 0.44	13.94 ± 1.88	8.66 ± 0.91
1800	0.9% Saline, I.P.	6	2.53 ± 0.73	11.80 ± 2.52	9.17 ± 1.00
0600	AOAA, 50 mg./Kg., I.P.	6	3.37 ± 0.82*	14.37 ± 4.57	11.68 ± 2.73
1800	AOAA, 50 mg./Kg., I.P.	5	3.32 ± 0.91	12.38 ± 3.33	8.45 ± 3.26
0600	AOAA, 70 mg./Kg., I.P.	7	3.69 ± 0.41***	16.36 ± 1.62**	10.02 ± 1.48
1800	AOAA, 70 mg./Kg., I.P.	7	3.56 ± 0.74*	16.46 ± 2.22**	10.72 ± 2.66

CHAPTER FIVE

GENERAL DISCUSSION

A. Possible Relationships of Circadian Rhythms in the Levels of Putative Inhibitory Transmitters to Physiological Parameters

Several types of 24-hour patterns in the concentrations of the putative CNS neurotransmitter substances studied can be described. These include: 1) Type I: a circadian pattern with a maximum in the dark phase as seen with the levels of DM and NE in the CS, US, and LS, of 5-HT in the lower brainstem, of GABA in the cerebral cortex, cerebellum, and corpora quadrigemina, and of glycine in the pons and spinal cord regions examined. This pattern was also obtained with rectal temperature, and motor activity, 2) Type II: a circadian pattern with a maximum in the light phase as noted in the case of the 5-HT levels of the corpus striatum and upper brainstem, 3) Type III: a "sawtooth" fluctuation apparently superimposed on a circadian pattern as noted in the case of the GABA levels of the thalamus-hypothalamus, 4) Type IV: a relatively stable pattern noted in glycine levels of the medulla.

1. Dopamine

The DM content of all examined tissues exhibited a Type I circadian pattern (Figures 11 and 12). This pattern differed from that described by Scheving and co-workers

(1968a), who found that DM content of the whole rat brain varied in an ultradian fashion. It is apparent from this difference that DM rhythms noted in the specific brain areas studied are not typical of all brain regions.

The brain regions used for this study of circadian DM content were selected because they represent a probable site of adrenergic pathways. The corpus striatum, which plays an important role as a "relay center" in the extra-pyramidal motor system (Crosby et al., 1962), was shown to contain extremely high concentrations of DM (Carlsson, 1959). Subsequent studies demonstrated the presence of a nigro-neostriatal pathway, which influences DM levels of the corpus striatum (Anden et al., 1964). A transmitter role for DM in this region has been proposed because DM fulfills certain necessary criteria: the presence of degradative enzymes (Bogdanski et al., 1957; Axelrod et al., 1959), the presence and accumulation of DM in the varicosities of fine neurons (Fuxe et al., 1964), an inhibitory action of DM on spontaneous firing induced by DL-homocysteic acid (Bloom and Salmoiraghi, 1965; York, 1967), in vitro DM release from DM-storing vesicles isolated from porcine caudate and lentiform nuclei (Philippu and Heyd, 1970) and in vivo DM release from the cat caudate nucleus (Riddell and Szerb, 1971). In light of the circadian changes in DM content in

this region, it is tempting to consider that these circadian fluctuations are occurring concomitantly with changes in the overall level of inhibition originating in this area. This supposition becomes more plausible when one considers that trough levels of DM are noted during the sleeping phase of this nocturnal species, a time when motor activity is at a nadir (Scheving et al., 1968a).

The adrenergic pathways that have been mapped in the upper and lower brainstem appear to be noradrenergic rather than dopaminergic (vide infra). The role of DM in these tissues is probably that of a precursor rather than a transmitter, and its fluctuation reflects circadian fluctuations in enzyme activity (Figure 13). The tyrosine hydroxylase step of catecholamine biosynthesis has been shown to be the rate-limiting step (Spector et al., 1965). McGeer and McGeer (1966) demonstrated that the activity of this enzyme is maximal in the rodent pineal during the dark phase. Thus, regardless of DM'S role in neurotransmission, its levels could be increased by augmented nocturnal tyrosine hydroxylase activity. However, it is also possible that increased DM content noted during the dark phase reflects an increased content of DM precursors. In this regard, Wurtman and co-workers (1968) have noted a circadian rhythm in the levels of phenylalanine and tyrosine in blood. In

actuality, both factors mentioned could be coupled to set the pattern in DM content.

An additional controlling factor in the regulation of DM levels could be attributed to the activity of the degradative enzymes of catecholamine biosynthesis. Piepho and Friedman (1968) demonstrated an inverse relationship between the striatal levels of DM and its primary metabolite, HVA (cf. Appendix B). The aforementioned study suggests that fluctuations occur in the activity of either MAO, COMT, or both.

The pharmacological implications of this rhythm in DM content are of considerable interest. Oxotremorine, a parkinsonomimetic agent that alters striatal DM content (Friedman and Anton, 1967), exhibits peak toxicity at noon in animals on a similar 12:12 light/dark regimen (Walker, 1969). The peak toxicity of this substance occurs at a time when the striatal DM content is minimal, thus re-emphasizing the role of DM in the parkinsonomimetic action of oxotremorine. More recently, Speciale and Friedman (1971) noted a circadian pattern in the sleeping time following a 350 mg./Kg. dose of the hypnotic agent, gamma-butyrolactone (GBL). Increased levels of CNS DM have been noted following treatment with this agent (Roth and Suhr, 1970; Aghajanian and Roth, 1970). However, the maximum

sleeping time noted with GBL occurred at 1800 hours, a time when the DM content of the brainstem and corpus striatum is minimal. It appears that if the action of GBL is directly related to DM content, this action is exerted in a brain region other than those studied. However, GBL also effects carbohydrate metabolism (Leonard and Watkinson, 1971), and this effect may be involved in the mechanism of its hypnotic action (cf. Discussion, V-A-4).

2. Norepinephrine

The NE content of brain regions studied varied on a Type I circadian basis (Figure 13). Friedman and coworkers (1968) demonstrated a similar pattern in the rat caudate nucleus and midbrain; this rhythm has recently been confirmed in the hypothalamus and caudate nucleus (Walker et al., 1971). The latter workers also noted a correlative circadian pattern in the number of granular catecholamine-storing vesicles in the pre-synaptic nerve terminals of these areas.

The pattern of striatal NE content has a time course similar to that of DM in this tissue. A functional role of NE in this area has been questioned because of the relatively small amount of NE found here (Bertler and Rosengren, 1959); however, NE may have a definite role in this region and may be synthesized as required (Phillis, 1970).

Regardless of its actual role, it is apparent that this NE rhythm follows that of DM. The conversion of DM to NE proceeds readily in the striatum due to high dopamine-beta-oxidase activity there (Kaufman and Friedman, 1965). The striatal NE rhythm might be attributed to the interference of high DM concentrations in the fluorimetric assay of NE. However, using the Ansell and Beeson technique, comparable levels of a DM standard solution caused no increase in fluorimetric readings over that of a reagent blank when treated and quantified in the NE procedure. Thus, the rhythm is not an artifact of the analytical method.

The twenty-four hour patterns of brainstem NE content also represent Type I patterns. The upper brainstem rhythm is more marked than that of the lower brainstem. This relationship is similar to that noted for DM in these regions. The hypothalamus contains the highest NE levels of the CNS (Glowinski and Iversen, 1966). In vivo release of NE has recently been demonstrated in the cat hypothalamus (Philippu et al., 1970). This area has been implicated in the control of body temperature by the work of Feldberg and his co-workers (1964), who found that application of NE close to the cat hypothalamus decreases body temperature. The present study in rats indicates that peak NE levels are found at a time when body temperature is also maximal,

which would seem at odds with Feldberg's finding. However, the measurement of total tissue levels of NE does not rule out the possibility of a highly localized adrenergic control of temperature, which could not be detected by the quantitative methods used in the current study. Furthermore, localized applications of neurotransmitter substances produce sudden changes in concentration in the face of endogenous transmitter currents, and the time course of these effects is distinct from those observed in circadian studies.

Noradrenergic mechanisms have also been implicated in the central regulation of food intake in the hypothalamus (Grossman, 1962; Booth, 1968). The NE rhythm found is in keeping with the postulated role of this area in satiety.

Hillarp and co-investigators (1966) demonstrated several noradrenergic pathways in the upper brainstem area. Ascending neurons of group A1 extend rostrally in the reticular formation to the median forebrain bundle, hypothalamus, and parts of the limbic system. These pathways appear to be important in the function of the waking animal, and higher NE concentration would be necessary in these areas during the waking hours to maintain the alert state.

The NE rhythm of the lower brainstem (pons-medulla) also peaks during the phase of maximal motor activity.

Fluorescence microscopic techniques have also been used to map noradrenergic systems of the lower brainstem. Hillarp and co-workers (1966) found that the majority of NE-containing nerve cell bodies are found in the pons and medulla. Descending neurons of group A2 extend caudally from the ventrolateral reticular formation via the anterior and lateral funiculi. These fibers give rise to NE-containing nerve terminals in the spinal cord (Dahlstrom and Fuxe, 1964). Some possible roles of NE in motor function have been described (a review of these spinal cord actions of NE is available, cf. Phillis, 1970). The increased NE content of the pons-medulla during the dark phase is concurrent with the peak of locomotor activity in these nocturnal animals. This result is not unexpected when one considers the physiological implications of the research of the Swedish workers regarding these descending pathways.

Control of these NE rhythms might be attributed to biochemical mechanisms similar to those described for DM (cf. Discussion, V-A-1). The time course of the NE rhythms in this study is similar to that of rat pineal gland NE (Wurtman et al., 1967). The latter rhythm is thought to be controlled by light impulses impinging upon the retina, and it is abolished by blinding. The actual biochemical control of the rhythm in the current study could be similar

to the mechanism controlling the pineal rhythm, viz., alterations in tyrosine hydroxylase activity. Another possible mechanism of control for the fluctuations of NE might lie in altered activities of the degradative enzymes, MAO and COMT.

The pharmacological significance of the NE rhythm is demonstrated by studies such as that of Black and co-workers (1969). They found that the depletion of NE in the rat brain by reserpine varies in a circadian fashion, with a greater NE depletion occurring during the dark phase. This is plausible if one considers that depletion of NE by reserpine occurs via a decreased granular binding capacity. Since more NE is available in the dark phase, a larger amount will be unbound by reserpine leading to a greater percentage of depletion. Studies of this type aid in clarifying one source of biological variation, so that measures can be taken in future scientific protocol to limit variability of this nature.

3. Serotonin

The patterns of 5-HT content in the corpus striatum and upper brainstem exhibited an inverse relationship to those noted for the catecholamines, viz., a Type II circadian pattern as seen in most 5-HT studies in rodents

(cf. II-B-6). In contrast, the 5-HT content of the lower brainstem exhibited a Type I circadian pattern similar to that obtained with the catecholamines (Figure 14).

The striatal 5-HT rhythm is similar in time course to that previously described in this laboratory (Friedman and Walker, 1968). The corpus striatum has the highest activity of tryptophan-5-hydroxylase (Peters et al., 1968), the rate-limiting enzymatic step in 5-HT biosynthesis (Garattini and Valzelli, 1965). However, recent reviews of CNS neurotransmitter activities fail to mention any action of 5-HT on the neurons of this region (Curtis, 1969; Phillis, 1970). Dahlstrom (1969) has described an ascending pathway from the pons-medulla that terminates in the striatal region, but the function of this pathway is not specified. It is plausible that this latter pathway could be a part of the trophotrophic system, in which 5-HT is the postualted transmitter (Brodie and Reid, 1968). This system, when activated, leads to increased parasympathetic output, drowsiness, sleep, and decreased motor activity; all of these parameters are maximal during the sleeping phase in mammals. The present study demonstrates that these functions attributed to the trophotrophic system do occur when 5-HT levels are maximal in the CS.

The 5-HT rhythm of the US is quite similar to that

of the CS. The peak in 5-HT levels occurs at a time when body temperature and motor activity are minimal. The possible roles of 5-HT in the various stages of sleep have been described by several authorities (Koella and Czicman, 1966; Koella, 1968; Pujol et al., 1971). Koella (1968) postulated that 5-HT receptor sites are present in the vicinity of the area postrema; once stimulated, ascending hypnogenic pathways, presumed to be serotonergic, are activated. An ascending serotonergic pathway has been demonstrated histochemically; it ascends from the rostral raphe nuclei to the diencephalon and telencephalon (Fuxe et al., 1968). The diurnal increase in 5-HT levels of this area coincides with the sleeping phase of rats.

In addition to the aforementioned functional role of 5-HT in the US, Scapagnini and co-investigators (1971) postulated that serotonergic pathways in the limbic regions play a role in the regulation of pituitary-adrenal function. A parallelism has been described in the rhythms of 5-HT content and plasma corticosterone in the cat. It is also interesting to note that US 5-HT levels peak when body temperature is minimal (cf. Figures 9 and 14). This seems to be in contrast to the relationship suggested by Feldberg and Myers (1964) which propose a direct relationship

between 5-HT levels and body temperature. However, Bruinvels (1970) has demonstrated that intra-cisternal injection of 5-HT produces a dose-dependent hypothermia. This latter study is in better agreement with the 5-HT data of the current study than is the data of Feldberg and Myers (1964).

The 5-HT rhythms of the LS exhibit a Type I pattern similar to that of the catecholamines of this area, (Figures 12, 13, and 14). This 5-HT rhythm is in marked contrast to those obtained more rostrally in the CNS (Quay, 1968; Friedman and Walker, 1968). Dahlstrom and Fuxe (1964) demonstrated the presence of 5-HT terminals in the motor nuclei of cranial nerves V and X, and in the nucleus of the tractus solitarius; they also describe a descending system between the caudal raphe nuclei and the lumbosacral areas of the spinal cord. In view of the probable role of such a system in motor function, a pattern of maximal levels during the period of greatest motor activity is not surprising (Figure 10). The depressant actions of 5-HT on dorsal root potentials, spinal cord reflexes, and interneuronal pathways have been recently reviewed (Phillis, 1970).

Control of the 5-HT rhythms could involve one or more of the following mechanisms. It could occur via the

pineal gland, which exhibits a rhythm in 5-HT content similar to that noted in the CS and US (Quay, 1963a). This pineal rhythm appears to be regulated via alterations in the level of 5-HTP decarboxylase, the activity of which varies according to the amount of light in the environment (Snyder et al., 1965a&b). The 5-HT rhythm of the LS could be regulated by the pineal rhythm of melatonin, which has a similar time course. This latter rhythm appears to be controlled by alterations in the activity of HIOMT, which is inhibited by light (Axelrod et al., 1965). One can postulate that the 5-HT rhythm of the LS might also be controlled by a fluctuation of MAO activity (vide supra), or by an alteration in the dietary intake of tryptophan, as increased plasma tryptophan has been shown to increase brain tryptophan concentrations, which in turn leads to increased brain 5-HT levels (Fernstrom and Wurtman, 1971).

4. 4-aminobutyric Acid

The only biorhythm that has been described so far for GABA levels was demonstrated in 1969 (Piepho and Friedman, 1969). This previous report described circadian and ultradian patterns in the CQ and TH-HT respectively (vide infra).

GABA levels of the frontal cerebral lobe exhibit a circadian pattern in which the difference between peak and

trough was of borderline statistical significance (cf. Figure 15, Appendix A). The rhythm however, is reproducible and was demonstrated in two different seasons. The nocturnal maximum of this rhythm coincides with the peak of motor activity in the rat (Scheving et al., 1968). The inhibitory actions of GABA on cortical neurons have been amply demonstrated (Krnjevic and Schwartz, 1967; cf. Bradley, 1969); increased levels of this inhibitory substance in this area could be anticipated with increased motor function, since motor pathways are associated with the frontal cortex (Crosby et al., 1962). On the other hand, one might ask "why GABA content is not elevated during sleep". Jasper and co-workers (1965) demonstrated a positive relationship between GABA levels in the pia-arachnoid space and the sleeping state. Since the brain preparation used in this study involved a saline rinse prior to dissection, some of this "released" or unbound GABA, present during the sleeping state, would have been rinsed from the tissue surface. This procedure might have distorted the true nature of the tissue rhythm, and a more accurate picture would probably be obtained if tissue levels and ventricular fluid content were both measured.

GABA levels of the CBLM exhibit a more definitive rhythm than those of the cortex, but have a similar time

course (Figure 15). The function of the cerebellum in motor coordination has been well established, and the biosynthesis of GABA has been confirmed in this structure (vide infra). Levels of GABA are quite high in the axons of cells with proposed inhibitory function, viz. Purkinje, basket, stellate, and Golgi II cells. The content of GAD, the immediate synthetic enzyme of GABA synthesis, is also higher in the Purkinje cell layer (Roberts and Kuriyama, 1968). The catabolic enzyme of this pathway, GABA-T, also exhibits highest activity in the neuronal areas receiving inhibitory synapses, viz. Purkinje, and Golgi cells, and fiber terminals (Roberts and Kuriyama, 1968). The inhibitory actions of GABA in this region have recently been reviewed (Phillis, 1970); and its release has also been documented following stimulation of afferent tracts (Obata and Takeda, 1969). In view of the inhibitory role of GABA in this motor center, a rise in levels, concomitantly with augmented inhibition associated with motor activity in the waking phase, is not unexpected.

The GABA rhythm of the corpora quadrigemina (Figure 16) is similar to that of the cerebrum and cerebellum, ie. a Type I pattern. Relatively high GABA levels have been reported in this structure (Fahn and Cote, 1968), suggesting a possible inhibitory function of GABA associated with

this region. The functions of the superior and inferior colliculi involve, respectively, the integration of visual and auditory impulses. Alteration in the level of inhibition in these structures might be anticipated due to the need for discrimination of pertinent stimuli during the waking phase. The GABA levels of the TH-HT exhibited a unique rhythm (Type III) that appeared as a "sawtooth" fluctuation superimposed on a circadian pattern (Figure 16). This type of fluctuation might seem plausible in light of the multiple functions of this area (Harris, 1966). These include regulation of body temperature, responsiveness to painful stimuli, maintainence of alert behavior, modulation of the pituitary axis, control of the drive for food and water, partial input into the physical expressions of emotion, and autonomic regulation. Although a circadian pattern might occur in some of the aforementioned functions, the homeostatic nature of others might require patterns with shorter periods and increased frequencies. An additional consideration concerns the possible role of GABA in modulation of monoamine activity, since the monoamines NE and 5-HT are found in high concentrations in this area (Glowinski and Iversen, 1966; Bogdnaski et al., 1957). Yessaian and collaborators (1969) demonstrated in vitro that GABA releases NE from hypothalamic slices and inhibits

5-HT release.

The central mechanisms of GABA rhythm control could be concerned with several parameters. Dietary intake of either glucose or pyridoxine (vitamin B_6) could affect GABA biosynthesis, as GABA is the product of an oxidative pathway with an anabolic B_6-catalyzed enzyme, GAD (Roberts, 1960). Tower (1960) demonstrated that virtually all of the energy metabolism of the CNS depends on the oxidation of glucose, the levels of which vary on a circadian pattern (Bahorsky and Bernardis, 1967). The latter investigators noted in the rat that the plasma glucose levels are highest during the dark phase, presumably due to increased feeding; levels of GABA are also maximal during this phase. Since vitamin B_6 is intimately involved in the GABA shunt (Roberts et al., 1964), the possibility of a feeding rhythm could also be implicated as a controlling factor. This supposition becomes more plausible in light of recent information that mouse brain GAD activity is higher in the dark phase than in the light (Roberts, personal communication). The coincident variation of a coenzyme could be involved in this difference in enzyme activity.

There has been some discussion in the literature concerning the mechanism of action of gammabutyrolactone, a sedative agent. Aghajanian and Roth (1970) have proposed

that dopaminergic pathways are responsible for the pharmacological effects of GBL, but that a time lag appears between maximal effect on DM content and the sedative response. More recently, Leonard and Watkinson (1971) have reported that gammahydroxybutrate, the active metabolite of GBL, caused an increase in brain glucose levels by any or all of the following factors: increased glucose synthesis, decreased glycolysis, or decreased amino acid synthesis from glucose. The circadian pattern of GBL sleeping time (Speciale and Friedman, 1971) would indicate that altered carbohydrate metabolism is more important than DM in GBL's action, since the DM rhythm was 180° out of phase with that of GBL sleeping times. In other words, DM levels are lowest when GBL sleeping time is longest. However, the GABA content is also lowest at maximal GBL sleeping time; thus, it appears that when synthesis of this amino acid is minimal, sleeping time is extended.

The rhythm in GABA levels also seems to affect the convulsant actions of allylglycine (cf. discussion, section V-D-1a).

5. Glycine

Glycine content exhibited different patterns in the individual brain regions examined (Figures 17 & 18). Type

I patterns were noted in the pons (Figure 17) and thoracic cord (Figure 18); the glycine content of these areas varied in a circadian fashion with a sharp peak late in the dark phase. The latter pattern has also been demonstrated for blood glucose levels in the rat (Friedman and Walker, 1969), with the peaks of both rhythms occurring coincidentally. Geiger (1958) found that brain glucose levels are proportional to those of the blood, and that the brain levels of free amino acids are, in turn, proportional to brain glucose. Shank and Aprison (1970) reported that glycine synthesis in the CNS occurs predominently via serine conversion catalyzed by serine hydroxymethyl transferase (EC 2.1.2.1). Furthermore, they demonstrated that glucose is the principle source of the carbon units of serine in the rat CNS. Thus, it is likely that the glycine patterns noted in the pons and TC reflect the pattern described by blood glucose levels, a pattern defined initially by glucose intake in the feeding cycle of the rat.

The glycine content of the medulla was relatively stable, exhibiting a Type IV pattern, in contrast to glycine patterns found in other CNS tissues examined. It is possible that regulation of a steady state level of glycine in this tissue occurs via interconversion to serine, the common storage form of glycine (Blakley, 1954; Kawasaki

et al., 1966). This stability of medullary glycine levels might be indicative of its association with a non-fluctuating medullary function. Indeed, glycine has been shown to exert a potent inhibitory influence on neurons of the cuneate nucleus (Galindo et al., 1967), which with their projection systems, function in the transmission of proprioceptive and tactile impulses. It is quite possible that these systems are maintained during both sleeping and waking phases with a fairly constant level of inhibition.

Fluctuations of glycine content of the cervical and lumbar spinal enlargements described a Type I circadian pattern (Figure 18). The pattern in these areas was not as sharp as that defined by the pons and TC, but the range in levels was still significant. The proposed role of glycine as a spinal inhibitory transmitter has been reviewed (Aprison and Werman, 1968; Werman and Aprison, 1968). These investigators have described higher glycine levels in the spinal cord enlargements relative to the mass of musculature supplied by these regions (Aprison et al., 1969b). Glycine has also been show to possess an equilibrium potential similar to that of the physiological interneuronal inhibitory transmitter indicating that they are probably the same (Curtis et al., 1968a). The circadian changes in glycine content parallel those of motor activity as

might be anticipated due to the greater need for regulatory modulation during movement. This regulation would require some specialized control mechanisms to prevent extreme fluctuation as noted in the pons and TC. Some of the mechanisms controlling glycine content in these areas include interconversion to serine (vide supra), incorporation of glycine into protein (Mase et al., 1962; Sky-peck et al., 1966; Aprison et al., 1969a), active uptake of glycine from the CSF (Aprison et al., 1969a), and active transport of glycine from CSF into blood (Murray and Cutler, 1970). Any or all of the aforementioned controlling mechanisms might aid in maintaining the constancy of glycine levels during motor activity, but it is not now possible to assign the degree of contribution of each factor. Thus, although large fluctuations in glycine content occur in the pons and TC where glycine probably functions only in intermediary metabolism, less marked variations are noted in the cervical and lumbar enlargements, where it appears to play a role in motor inhibition.

The pharmacological implications of these rhythms can be appreciated when one examines the data on the circadian toxicity of strychnine, a glycine antagonist (cf. discussion section V-D-1b).

B. Effects of Photoperiod Reversal on the Levels of Putative CNS Neurotransmitters

The technique of photoperiod reversal was utilized in this study to evaluate both the endogenous or exogenous character of the rhythm and the effect of reversal as a stress on cumulative amine levels. Exogenous, or extrinsically regulated rhythms were noted in the striatal DM, NE, and 5-HT content, upper brainstem 5-HT content, and cerebral and cerebellar GABA content. Endogenous, or intrinsically controlled rhythms were demonstrated in catecholamine content of both brainstem regions. In other rhythms investigated (5-HT of the lower brainstem, collicular GABA, and GABA of the TH-HT), reversal appeared to cause a flattening of the pattern seen on a normal cycle.

1. Dopamine

The pattern of DM levels in the corpus striatum appeared to be exogenous as it was readily reversed by photoperiod reversal (Figures 19 & 20). The exogenous nature of this rhythm was not surprising in light of the relationship of DM to the extrapyramidal motor system (cf. discussion, section V-A-1). Thus, striatal DM levels apparently peak during the phase of maximal motor activity regardless of the light/dark cycle used. On the other hand, the rhythms

in DM content of both brainstem areas were found to be endogenous (Figure 20). It is difficult to ascribe a function to these latter patterns because of the limited number of studies dealing with the effect of photoperiod reversal on physiological functions. However, it should be noted that brainstem NE, which is derived from DM, is also endogenously regulated.

A comparison of the striatal DM content in the comparative phases of the two experimental cycles reveals that photoperiod reversal leads to increased basal DM content (Table 2). In the pilot study on DM content (Figures 11 & 19), an increased basal level of DM was noted under reversal conditions. Since the possibility of seasonal variation existed in this initial study, a second study was performed with rats conditioned on both cycles simultaneously (Figures 12 & 20). Striatal DM levels were once again higher on the reverse cycle, suggesting the possibility of a stress-induced increase. Although DM levels are stable under some types of stress, viz. foot-shock stress (Bliss et al., 1968), oscillatory stress (Smookler and Buckley, 1969), and aggregation (Bliss and Ailion, 1969), other forms produce increased DM levels. Welch and Welch (1969) noted increased DM levels due to a chronic fighting stress. Furthermore, NE turnover has been found to increase in a

variety of stress situations (cf. discussion, section V-B-2), suggesting that an increased level of DM would be needed to augment NE biosynthesis.

A comparison of brainstem DM content during the activity or sleep phases reveals an interesting difference in the effect of photoperiod reversal on this tissue. The DM content of the lower brainstem is increased during inversion, but that of the upper brainstem is not. The action of photoperiod reversal on lower brainstem DM might produce a stress similar to that described for the CS. The lack of any apparent effect on the US suggests that this brainstem area responds differently to "inversion stress", but the reasons for this localized specificity in response are unclear. It is unfortunate that many studies dealing with stress involve catecholamine levels of the whole brain, or brainstem *in toto*, rather than individual regions of the brain. Localized responses of the type demonstrated in this study might occur, but are obscured by homogenization of the whole brain.

2. Norepinephrine

Photoperiod reversal has similar effects on the NE patterns as on those of DM (Figure 21). The reversal of the striatal pattern of NE is not surprising, as NE levels of this tissue probably follow those of DM (cf. discussion,

section V-A-2); thus, the reversal of the DM rhythms (vide supra) is of prime importance in the NE rhythm.

The NE rhythms of the brainstem were unchanged by photoperiod reversal indicating an endogenous character. These endogenous rhythms might play a role in some non-reversible endocrine or autonomic function, since the noradrenergic pathways of the brainstem extend rostrally to the level of the diencephalon (Hillarp et al., 1966). The question of defining a non-reversible parameter is a difficult one since most studies indicate reversibility of hypothalamically-controlled functions, viz. body temperature (Figure 9), locomotor activity (Figure 10), and ACTH release (Krieger, 1970). It is also interesting that one of the previously postulated control mechanisms, pineal influence, would not appear to control this rhythm, as the NE rhythm of the pineal gland is affected by photoperiod alterations (Wurtman et al., 1967). Both the control mechanisms of, and the parameters affected by this rhythm offer fertile areas of future study.

Cumulative striatal NE levels are significantly higher during the phase of activity of the reverse cycle than during this phase of the normal cycle. This could indicate a stress factor associated with photoperiod reversal, as has been previously described for DM content

(vide supra). Changes in NE turnover have been associated with several stressful situations, viz. intense muscular exercise (Gordon et al., 1966), immobilization (Corrodi et al., 1968a; Welch and Welch, 1968), changes in environmental temperature (Corrodi et al., 1968b; Simmonds and Iversen, 1969), chronic electroshock (Kety et al., 1967), and foot-shock treatments (Thierry et al., 1968; Bliss et al., 1968).

Two alterations in cumulative NE levels were produced by photoperiod reversal: increased minimal NE levels in the US, and increased maximal NE content in the LS. The reason for these localized increases in NE content on the reverse cycle remains obscure, but some stress factor in local brainstem pathways could explain these alterations. It is quite possible, if these rhythms are assumed to be due to increased enzyme activity, that the increased NE levels during the phase of maximal NE content in the LS merely reflect the higher DM levels of this tissue during reversal.

3. Serotonin

The effects of photoperiod reversal on 5-HT content involve reversal of the 5-HT rhythms of the CS and US, and a flattening of the pattern in the LS (Figure 22).

Striatal 5-HT levels are reversed so that their maximum still coincides with the troughs of motor activity, or the sleeping phase of this nocturnal species. Although the role of 5-HT in this area is unclear, it is evident that levels follow the pattern of those in the upper brainstem, which appear to be involved in sleep mechanisms (cf. discussion, section V-A-3).

A similar reversal occurs in the pattern of 5-HT content in the US. This reversal strengthens those theories that relate 5-HT content to sleep, as reversal of the sleep/wakefulness cycle also occurs. Thus, maximal 5-HT content is still noted during the sleeping phase. The control of these rhythms of the CS and US could be seated in the pineal gland, which exhibits a similar rhythm determined by fluctuations in the activity of 5-HTP decarboxylase (Snyder et al., 1965a).

The 5-HT pattern of the LS, which was 180° out of phase with the other 5-HT rhythms on the normal cycle, seemed to disappear when subjected to reversal. The abolition of the rhythm offers an intriguing possibility, that coinciding exogenous and endogenous patterns are present. These patterns might be in phase on the normal cycle, but out of phase on the reverse cycle. This possibility gains credence when one considers that Quay (1968) has reported

differential 5-HT rhythms in the brainstem, an exogenous one in the lateral brainstem and the absence of any 5-HT rhythm in the medial brainstem. An additional consideration involves the different pathways described by Dahlstrom and Fuxe (1964). They demonstrated serotonergic pathways from the vasopressor region of the rat medulla to the sympathetic lateral column of the spinal cord, and an ascending pathway to the median forebrain bundle. Differential pathways of this type could possess differential rhythms depending on their function; thus, it is possible that the apparent loss of this rhythm actually represents the effect of a reversed photoperiod on an exogenous rhythm, and its lack of effect on an endogenous one.

A comparison of the cumulative 5-HT levels of comparable phases indicates only one significant difference (Table 4) - an increased 5-HT trough level in the LS on the reverse cycle. This increase further emphasizes the possibility of an endogenous 5-HT rhythm in this tissue, which resists reversal and overrides the effects of photoperiod inversion. The lack of any other alteration in 5-HT content is not surprising since other studies of stress have indicated that 5-HT levels are essentially unchanged (Bliss et al., 1968; Bliss and Ailion, 1969; Welch and Welch, 1969).

4. 4-aminobutyric Acid

The effects of photoperiod reversal on central GABA levels involved some differential inversion patterns dependent on the tissue under study. The inversion of the pattern in GABA content was marked in the frontal cerebral lobe and cerebellum (Figure 23). This reversal parallels that of motor activity, and such a response might be expected in view of the potential roles of GABA previously described (cf. discussion, section V-A-4). The reversed rhythm also coincides with the feeding rhythm, so that the dietary intake of glucose and pyridoxine might also be involved in regulation of this reversed pattern. It is also possible to speculate that some of the enzymes involved in oxidative metabolism in these areas might express photodependent changes in activity.

A "pure" circadian rhythm could not be demonstrated in the CQ following photoperiod reversal, although a pattern with marked peak and trough was noted (Figure 24). The peak and trough, however, both occurred in the dark phase, possibly indicating a greater resistance to the effects of reversal in this tissue. The reason for the increased resistance can only be speculated upon, but it might be related to the high concentration of GABA in this tissue (Fahn and Cote, 1968), and the degree to which the

enzymatic activity adapts to the new photoperiod. It may be that adaptation of a sufficient percentage of the total enzyme content necessary for a clear demonstration of inversion does not occur in the limited three week period of adaptation commonly used. The possibility of differing adaptation periods for specific rhythms, depending on the tissue level of transmitter and the controlling factors involved, must be considered in studies of this type.

The "sawtooth" fluctuation of TH-HT GABA content previously described resists photoperiod reversal, although the circadian component of this rhythm appears to be reversed (Figures 24 & 25). This apparently is an example of an extreme fluctuation occurring in the presence of an exogenous circadian pattern. It is quite possible that more than one type of rhythmic pattern in the levels of this putative inhibitor could occur in a multi-functional area as the hypothalamus (Harris, 1966). It appears as though patterns of different periods may be synchronized on the normal cycle, but become desynchronized on the reverse cycle as the ancillary fluctuations are less clearly defined following inversion (Figure 25).

When the cumulative GABA levels of various phases are compared (Table 5), significant increases in GABA content of the normal cycle are noted in the CQ and the TH-HT.

However, the significance of this finding is suspect, since the studies on these two areas were conducted during different seasons. Seasonal fluctuations in GABA levels have been reported (Roberts, personal communication) due to seasonal differences in the pyridoxine content of various rat food formulae. These diets are made from grain sources which have different vitamin content depending on environmental factors, eg. length of growing season, time of harvest, etc. A similar comparison of GABA levels of the frontal cerebrum and CBLM revealed only one significant difference - cortical GABA levels of the normal light phase are higher than those of the reverse light phase (Table 5). The range of values of cortical GABA levels was greater on the reverse cycle due to the fact that levels of the light phase had decreased leading to a significant difference between peak and trough not seen in the normal cycle. This definition of the reverse cycle GABA rhythm is puzzling. It is possible that GABA plays some hitherto unsuspected role in stress-related situations, but no studies of the effects of stress on central GABA levels have been found in the literature. Moreover, GABA levels are apparently affected only in the cortex (of those areas examined in the current study). A correlative study of catecholamine and amino acid levels of the cerebrum during stress might aid

in evaluating this regional difference.

C. Evaluation of Electroshock Convulsive Mechanisms During Different Phases

The effects of maximal electroshock seizure induction (MES) were evaluated in Ha/ICR mice in light and dark periods of the normal cycle. Since 0.9% saline solution and 5% gum acacia suspension were to be used as vehicles for the test drugs, it was important to determine whether they affect control mice in the same way (Table 6). The evaluation involved a measurement of the timing of the seizure components: extensor latency, tonic extension, and clonic phase. These phases were used by Tedeschi and co-investigators (1956) in their evaluation of the effects of current intensity on the timing of seizure components. At the 50 ma. level utilized in these studies, the seizure times were consistent with those of the former study.

The mechanisms underlying generalized tonic/clonic seizure patterns appear to be located in the spinal cord Esplin and Freston (1960) demonstrated tonic/clonic patterns following synchronous stimulation of the cervical spinal cord. They proposed that the motor expression of the seizure involved the "sterotyped response of spinal reflex systems to generalized stimulation of the cerebrospinal tracts". The initial response to a massive stimulus

is one of flexion because the flexor muscles are under a lower degree of inhibition than are the extensors. Tonic flexion leads to a lessening of inhibition on the more powerful extensors, thus allowing this dominant muscle group to contract resulting in tonic extension. The terminal clonic movements appear to depend on "interactions between the antagonistic motoneuron pools through inverse myotactic reflexes, as well as upon the nature of the cerebral seizure discharges which play upon the motoneurons". Gastaut and Fischer-Williams (1959) demonstrated that the caudal reticular formation is the critical brainstem area for transmission of seizure impulse traffic from brain to spinal cord. The mode of activation of the caudal reticular formation might involve either hypersynchronous activity or a release from higher central inhibitory mechanisms.

Circadian changes in certain types of seizure phenomena have been reported in various species of nocturnal rodents. The susceptibility to Indoklon-induced seizures has been shown to be greater in the dark phase in both mice and rats (Davis and Webb, 1963; Webb and Russell, 1966). Schrieber and Schlesinger (1971) noted a circadian pattern with peak susceptibility in the nocturnal phase to both sound-induced and electrically-induced seizures

in DBA/2J and F_1 hybrid mice. A similar rhythm of marginal significance ($P < 0.1$) was described in C57BL/6J mice. Wada and Askura (1970) have also described maximal susceptibility to audiogenic seizures in the Wistar albino rat during the nocturnal phase. Thus, peak susceptibility to these seizure induction procedures occurs during the dark phase in these nocturnal rodents.

The only diurnal/nocturnal difference that was noted in the current study utilizing control Ha/ICR mice involved a shortening of the extensor latency time in the dark phase (Table 6). A decreased latency time might be expected during the nocturnal phase, owing to the increased seizure susceptibility found in this phase (vide supra). Extensor latency time is essentially equal to the time of the tonic flexion phase. Since the levels of most excitatory and inhibitory putative transmitter substances are maximal at night, impulse traffic might be transferred more readily in this situation in which the animal is normally involved in motor function.

Some differences in seizure component times were also noted in comparisons of saline-treated versus acacia-treated control animals (Table 6). Extensor latency time in saline-treated animals was significantly shorter at 0600 hours, and the time of tonic extension was shorter in

saline-treated animals at both time points. Acacia was utilized in this study because of its previous use in evaluation of the anti-convulsant activity of meprobamate (Berger, 1954). The compound is commonly used for the suspension of insoluble substances in water; it is considered ideal because it exerts a similar osmotic pressure to that of serum protein (Martin et al., 1961). Gum arabic (acacia) consists mostly of arabin, the calcium salt of arabic acid; upon hydrolysis, it yields arabinose, galactose, and arabinosic acid (Martin et al., 1961). It appears that the components of this suspending agent, or perhaps the possible alteration of calcium levels, or some ionic binding properties of the gum might have an action on the timing of the tonic extension phase.

The lack of alteration in both seizure component times and minimal electroshock threshold suggest the possibility of a "homeostatic equilibrium" in the excitability of the CNS. As noted in the current study, levels of putative "threshold-elevating" substances (DM, NE, GABA, glycine) are maximal during the dark phase. Thus, it might be assumed that the threshold would also be higher in this phase, but it must be remembered that CNS excitability is based on the relative levels of both excitatory and inhibitory influences. The level of acetylcholine in

the rat brain is also maximal in the dark phase (Walker, 1969), and ACh has "threshold-lowering" actions (cf. Maynert, 1969). The increased ACh level might offset the increased levels of inhibitory substances leading to a stability in seizure susceptibility. It is also possible that the levels of another potential excitatory substance, glutamate, are elevated during the dark phase as the levels of glucose are maximal during this phase (cf. discussion, section V-A-5). The CNS levels of this amino acid transmitter suspect should be proportional to the level of free glucose. In conclusion, the constancy of most seizure component times as well as of minimal electroshock seizure threshold might be due to a balance between the excitatory and inhibitory neurochemical influences on the CNS. These substances appear to fluctuate in similar circadian patterns; thus, it appears that the measurement of any individual neurochemical component cannot serve as an index for the complex mechanisms involved in MES seizure production.

D. The Evaluation of Drug Efficacy in Response to Light or Darkness

1. Convulsant Compounds

a. Allylglycine

The peak toxicity of allylglycine was found to occur

at 1800 hours, late in the light phase (Figure 26). This peak coincides with minimal levels of GABA in the whole rat brain (calculated from the previously noted individual data), or minimal activity of GAD in the mouse brain (Roberts, personal communication). Allylglycine was shown to have convulsant properties by Schneider and co-investigators (1960). Alberici and collaborators (1969) demonstrated that the drug definitely inhibited GAD with no alteration in the activity of GABA-T (this action leads to a decreased level of GABA in the rat CNS). Allylglycine was found to have no effect on the metabolism of pyridoxine, aspartate, glutamate, glycine, glutamine, or alanine in the CNS. Administration of this convulsant agent further led to a marked structural alteration in the nerve endings of the cerebellar Purkinje cell layer.

The peak toxicity of allylglycine also occurs coincidentally with minimal glycine content in the lumbar spinal cord. Recent evidence presented by Roper (1970) has indicated a similar membrane receptor site for both glycine and allylglycine in the Mauthner cells of the goldfish. Thus, it is possible that a competition of a similar nature could occur in the mammalian spinal cord.

The minimal toxicity of allylglycine was noted during the dark phase, when levels of both GABA and glycine

are maximal. From these results, it is impossible to assess the relative importance of either transmitter suspect in the action of allylglycine; however, both compounds might be involved (vide supra). In contrast to electroshock, a circadian pattern was obtained with this convulsant agent. This response is probably seen because, unlike electroshock, allylglycine affects one, or possibly two, inhibitory systems. Thus, it is capable of upsetting the balance of excitatory and inhibitory CNS factors, resulting in a differential pattern of toxic actions.

b. Strychnine Sulfate

The circadian toxicity curve of strychnine sulfate is similar to that of allylglycine (Figure 26). Peak toxicity of strychnine was also noted at 1800 hours, when glycine levels of the lumbar spinal cord are minimal. The role of glycine as an inhibitory transmitter in this latter region has been extensively investigated (cf. Aprison and Werman, 1968). Strychnine has been shown to supress reversibly both post-synaptic inhibition in the spinal cord and the hyperpolarization of spinal neurons due to the iontophoretic application of glycine (cf. Curtis, 1969). Thus, it appears that the glycine levels of the spinal cord influence the toxicity of strychnine, a supposition borne out by a comparison of their respective circadian

patterns. In contrast to the actions of allylglycine, strychnine has been shown to have no actions on the effects elicited by iontophoretically-applied GABA (cf. Curtis, 1969), so the effects noted here can be more directly related to glycine content.

In contrast to the rhythm noted in this study, Tsai and co-workers (1970) reported a circadian toxicity curve for strychnine in which maximal toxicity occurred during the dark phase in rats on a programmed L/D cycle. It should be emphasized that these investigators did not isolate the strychnine-treated rodents, so that greater interaction would be expected during the dark phase. This enhanced interaction of animals housed together would lead to increased seizure susceptibility. The mice in the current study were isolated following strychnine injection (cf. Methods section, II-D-2-a), therby preventing the aggregation effect and allowing a truer appraisal of the pharmacological actions of the drug.

2. Anti-convulsant Compounds

a. Acetazolamide

The anti-convulsant action of acetazolamide did not vary appreciably when tested during different phases of the illumination cycle in either the mouse (Table 7) or the rat (Table 8). The anti-convulsant action of

acetazolamide has been postulated to occur via various mechanisms (cf. Millichap, 1965, 1969). The most investigated of these mechanisms involves the inhibition of brain carbonic anhydrase by acetazolamide which leads to increased CO_2 levels in the CNS, a decreased blood pH, and a decreased seizure susceptibility (Millichap, 1969). This increased brain CO_2 level is accompanied by an increased CNS ratio of extracellular/intracellular sodium ions, which stabilizes the neuronal membranes and limits aberrant discharges. Additional studies have demonstrated that acetazolamide decreases the uptake of radioactive sodium by brain cells (Millichap, 1965). It is difficult to evaluate the role of light programming on this mechanism, since the possibility of rhythms in brain carbonic anhydrase activity has not been investigated. However, the stability of dosage demonstrated would indicate that either carbonic anhydrase activity is relatively unvarying, or that acetazolamide is detoxified at a rate that varies in opposition to carbonic anhydrase activity. Acetazolamide is rapidly absorbed, well distributed and excreted mainly in an unchanged form in the urine (cf. Millichap, 1965). Thus, a circadian pattern in urinary excretion, which is greater in the dark phase in nocturnal species (Zsoter and Sebok, 1955; cf. Mills, 1966), might be expected to

increase acetazolamide clearance during this phase.

Two ancillary mechanisms for the action of acetazolamide involve putative CNS neurotransmitters. Woodbury and Esplin (1959) demonstrated that acetazolamide administration results in increased levels of glutamic acid, glutamine, and GABA. Thus, if the action of acetazolamide depends on GABA, a maximal response might be expected during the dark phase (when CNS levels of GABA are maximal). However, it most be remembered that excretion of acetazolamide might be maximal in the dark phase (vide supra), and would thereby "neutralize" the rhythmic fluctuations in response. Gray and Rauh (1971) indicated an involvement of the monoamines NE and 5-HT in the anti-convulsant action of carbonic anhydrase inhibitors. These researchers claim that a reduction of anti-convulsant potency accompanies depletion of the monoamines by synthesis inhibitors. However, more recently, Koslow and Roth (1971) have shown that monoamines are probably not involved in this action because the anti-convulsant action of acetazolamide was similar in reserpinized and control animals. The fact that NE and 5-HT rhythms are 180° out of phase with each other complicates any assessment of the role of monoamines in the anti-convulsant action of acetazolamide.

Acetazolamide increased the extensor latency time

over that of control animals in both phases of the illumination cycle (Table 9 & 10). This result was not surprising since similar changes are commonly noted in the laboratory evaluation of anti-convulsant agents (cf. Millichap, 1965). Acetazolamide also increased the time of clonus in mice during the light phase (when compared to control). This phenomenon is seen with other anti-convulsants, eg. diphenylhydantoin, and is said to be related to the ability of a compound to prevent the spread of seizure impulses (cf. Millichap, 1965 and Woodbury, 1969). The factors behind this increased clonic time in the mouse during the light phase remain unclear.

b. Diphenylhydantoin

As in the case of acetazolamide, there was no difference in the PD50 dose of diphenylhydantoin between phases in either species studied (Tables 7 & 8). Diphenylhydantoin is well absorbed, and is distributed readily to most tissues. Due to extensive protein binding, only 20-25% of the drug exists in the free form in plasma. A large percentage of the total dose (95%) is metabolized in the liver, with the major metabolite being the p-hydroxyphenyl derivative. The anti-MES activity of diphenylhydantoin is due to its ability to prevent the spread of the seizure by blocking post-tetanic potentiation

(PTP). The blockade occurs by stimulating "sodium pump" activity (Woodbury and Kemp, 1971). The lack of any circadian response is unexpected in view of the work of Radzialowski and Bousquet (1968), who noted that hepatic drug-metabolizing enzymes in the rat exhibit maximal activity during the dark phase. Thus, with an enhanced drug metabolism and an increased urine flow to remove the metabolized drug in the dark phase, a decreased potency could be anticipated. However, the higher levels of GABA in the CNS during the dark phase would tend to enhance the action of diphenylhydantoin. The drug has been shown to decrease the concentration of brain glutamic acid while increasing that of GABA (Vernadakis and Woodbury, 1960). More recently, Woodbury and Kemp (1971) postulated that the "hyperpolarization of nerve endings of inhibitory neurons by DPH could also increase release of inhibitory neurotransmitters". The increased nocturnal GABA content could lead to a greater inhibitory action and compensate for the enhanced degradation of the drug in this phase, dampening any circadian fluctuation.

When the seizure component times of diphenylhydantoin were compared with control, the following differences were noted: 1) increased extensor latency time in both species and during both phases, 2) increased clonic time during

the light phase in the rat, 3) decreased tonic extension time during both phases in the mouse and during the light phase in the rat (Tables 9 & 10). The increased extensor latency time and decreased tonic extension time would be anticipated in the case of diphenylhydantoin as these changes are noted with most anti-convulsants (cf. Millichap, 1965). The lack of a decreased tonic extension time in the rat during the dark phase suggests a decreased effectiveness of diphenylhydantoin, although the fluctuation of this component is not reflected in the PD50 comparisons. This supposition is reinforced by the decreased period of clonus seen in the rat during the dark phase. An increased period of clonus is characteristic of the hydantoins (cf. Woodbury and Kemp, 1971). This exacerbation of clonus has been attributed to a hydantoin-induced decrease in the level of pre-synaptic inhibition in various tracts involved with the production of clonic movements (Woodbury, 1969).

These fine changes in component timing might indicate a greater rhythmic fluctuation in the metabolism of diphenylhydantoin in the rat. This possibility is further emphasized by the significant decrease in tonic extension in the light phase as compared to that of the dark phase in diphenylhydantoin-treated rats. Thus, the comparatively prolonged tonic extension during the dark phase could be indicative of a lower efficacy at that time, although this

change is subliminal when only the PD50 is considered. A study of the circadian pattern in diphenylhydantoin metabolism and turnover could prove to be valuable in considering these aforementioned fine changes.

c. Meprobamate

In contrast to the two drugs previously discussed, the PD50 of meprobamate was significantly higher in the nocturnal phase in the mouse, and a similar difference of borderline significance was obtained in the rat (Tables 7 & 8). Meprobamate's anti-convulsant actions were first described by Berger (1954), although the drug is more commonly used as a sedative anti-anxiety agent (Domino, 1971). The drug is readily absorbed and uniformly distributed; 88-90% of the total dose is metabolized to either the hydroxy derivative (60%) or the glucuronide metabolites (30%). These metabolic changes occur in the liver, and their products are excreted in the urine (cf. Domino, 1971). The mechanisms of meprobamate action probably lie in its depressant actions on several CNS areas, viz. the thalamus, limbic system, reticular formation, hypothalamus, and spinal cord (cf. Smith, 1965). The higher PD50 values obtained in the dark phase might be due to the altered motor activity of this phase. Increased motor activity during this stage in the circadian cycle could have antagonistic effects on

the general depressant action of meprobamate. One additional factor involved in the nocturnal elevation of the PD50 would be an increased hepatic metabolism of the drug during this phase. Radzialowski and Bousquet (1968) demonstrated that the hepatic drug metabolizing enzymes have maximal activity in the dark phase in rodents. These combined factors probably lead to the altered efficacy of the drug seen in the dark phase.

Meprobamate increased extensor latency time and decreased tonic extension time in both species and all phases, when compared with controls (Tables 9 & 10). As discussed previously (cf. discussion, section V-D-2-a and V-D-2-b) these changes were anticipated, and confirm the anti-convulsant efficacy of this agent. The duration of the clonic phase was also increased during both phases in the mouse and during the light phase in the rat. This increased clonus with diphenylhydantoin and acetazolamide has been previously discussed (cf. discussion, section V-D-2-b); however, an action of the type noted for diphenylhydantoin has not been demonstrated for meprobamate. The enhanced clonus noted during the light phase in the rat might be indicative of the increased potency of meprobamate during this phase of diminished activity.

d. Phenobarbital Sodium

Phenobarbital in rodent species during both phases gave identical changes as those noted with diphenylhydantoin, viz. an unaltered PD50 value (Tables 7 & 8), with finer differences in the duration of rat seizure components (Tables 9 & 10). Phenobarbital is 77-89% metabolized in the liver via para hydroxylation of the phenyl ring. This inactive metabolite is excreted in the urine (Woodbury and Kemp, 1971). In addition, roughly one third of an administered dose of phenobarbital is bound to plasma protein (cf. Millichap, 1965). The anticonvulsant actions of phenobarbital are "multiple and rather nonselective as compared with the rather pure effects possessed by DPH" (Woodbury, 1969). Some of these effects include inhibition of oxidative metabolism, altered transport of sugars, uncoupling of oxidative phosphorylation (cf. Woodbury, 1969) and a decrease in the production of free acetylcholine (McLennan and Elliot, 1951). In consideration of phenobarbital's metabolism and mechanisms of action, a maximal effect might be expected to occur during the light phase of the cycle due to decreased hepatic metabolism (Radzialowski and Bousquet, 1968) and decreased urinary flow (Zsoter and Sebok, 1955). In addition ACh levels are lower at this time (Friedman and Walker, 1969b; Massarelli et al., 1970)

and oxidative metabolism appears to be minimal as reflected by the decreased GABA levels of this phase. The stability of the PD50 and phenobarbital's resemblence to diphenylhydantoin under similar conditions suggests that these aforementioned mechanisms are less important for phenobarbital's anti-convulsant action than the common action that it shares with diphenylhydantoin - an inhibition of post-tetanic potentiation (cf. Woodbury, 1969).

When seizure component times are considered, phenobarbital administration led to an increased extensor latency time in both species during both phases, and a decreased tonic extension and an increased clonic time during both phases in the mouse, and during the light phase in the rat (Tables 9 & 10). Once again, these alterations were similar to those of diphenylhydantoin, and might be due to similar mechanisms. The reason for the fine differences in the seizure component times of the rat during different phases is unclear, but might lie either in some difference in the metabolic pattern or in the degree of subliminal depression in these two species.

e. Amino-oxyacetic Acid

Amino-oxyacetic acid (AOAA) has been reported to be an effective anti-convulsant capable of abolishing the tonic extensor phase of electroshock seizures (Kuriyama

et al., 1966). Attempts to duplicate this action in the present study were unsuccessful, even though the trial doses ranged between the ED1 and LD40. There were only two apparent differences between the two studies: 1) the seizure induction method, 2) the type of light source used. Kuriyama and co-workers (1966) used skull electrodes, whereas corneal electrodes were used in the present study. These two methods of seizure induction are apparently different in their action, as the minimal electroshock threshold is 61 ma. in one (skull electrodes) and 10 ma. in the other (corneal electrodes). A plausible explanation of this difference might lie in the pathways involved in seizure induction. A shock administered via skull electrodes might initiate seizure by either direct stimulation of surface tissue layers or by a direct spread of the current across the skull or meningial linings to the spinal cord. Administration of shock via corneal electrodes probably initiates a seizure via supra maximal stimulation of afferent tracts; the seizure occurs due to transmission of this stimulus through the brain pathways to the spinal cord.

It is also possible that the difference in the quality of light source used could contribute to the differences noted in the data between the two laboratories.

Fluorescent lighting was used as a light source in the initial study (Roberts, personal communication), while full spectrum daylight (Vitalite) was used in the current study (cf. Figure 1). Although this difference might seem unimportant, the effects of altered spectrum of light can be marked in certain biological systems (cf. section II-A-2).

Two consistent findings were noted with higher doses (50-70 mg./Kg.) of AOAA: an increase in extensor latency and tonic extension times (Table 13). The increased extensor latency time is not surprising in light of the action of AOAA in increasing GABA content of the CNS (Kuriyama et al., 1966). The role of increased content of this putative inhibitory substance in seizure mechanisms has already been described (cf. section II-D-5). However, the increased duration of tonic extension is quite interesting as many drugs that prolong extensor latency time cause a decreased tonic extension time (vide supra). However, both tonic phases (flexion and extension) of MES seizure are enhanced by the drug. This prolongation of the tonic phase seems to be quite an anomalous response in light of GABA's inhibitory actions, and a study of the actual role of GABA in the timing of seizure components would prove to be a most informative and valuable study.

E. A Critique of the Use of Twenty-four Hour Rhythms in Research

Studies of the 24-hour fluctuations in the content of biochemically important substances are necessary for a true assessment of the dynamic relationships involved in homeostasis. Studies of this type allow the investigator to observe a dynamic rather than the static picture usually observed in studies done at a single timepoint. All too often, investigators observe static phenomena at a specified timepoint and fail to denote the time of their observation in their reports. Misleading estimates of parameters result when one attempts to evaluate data obtained by different research groups at undefined times. In contrast to the incomplete information gleaned from a single timepoint study, the knowledge of circadian patterns allows the investigator to establish dynamic interrelationships in biochemical and/or physiological systems, and permits him to select an optimal time for the study of a given system.

A certain amount of caution must be exercised in the interpretation of 24-hour fluctuations in biochemical levels since the content of the substance is not the only critical factor. The actual mechanisms occurring at the receptor level for these substances are also quite important, and measurement of the total content of a substance

yields little information concerning the intrinsic activity at a given time. Furthermore, the presence of simultaneous rhythms of different biochemical substances in mutually antagonistic or synergistic systems can obscure the specificity of a related physiological rhythm.

Studies in chronotherapeutics are just beginning to elicit useful information concerning diurnal and nocturnal drug dosage schedules. The relative importance of an appreciation of circadian rhythms in drug metabolism becomes apparent in the latter sections of the discussion (vide supra). Monitoring of the rhythms in drug responses allows for some speculation concerning the drug's relationship to biochemical parameters, and studies of this type might eventually aid in clarifying the actual mechanism of action of certain drugs. It is apparent from this study that drugs, which affect a specific neurochemical system, are more likely to follow the circadian fluctuations of that system than drugs, which have non-specific actions. This latter group appears to affect multiple neurochemical systems and their rhythms, if present at all, are more difficult to define due to the possibility of actions on many systems that might have differential patterns with differing maxima and periods. In conclusion, the current study indicates the need for further research in the area of chronopharmacology and chronotherapeutics.

CHAPTER SIX

SUMMARY

1. The study involved an initial adaptation of adult rats and mice to controlled laboratory conditions for three weeks prior to analyses of regional brain content of transmitter suspects or to evaluation of convulsant and anticonvulsant activity. Full spectrum lighting was used. Two lighting cycles that were 180° out of phase with each other were used to determine the endogenous or exogenous character of some of the measured parameters. The normal cycle lasted from 0800-2000 hours, and the reverse cycle from 2000-0800 hours.

2. The catecholamine (either DM or NE) content of various areas of the rat brain was found to vary in a circadian fashion. The levels of these amines in the corpus striatum varied in an exogenous circadian pattern, while that of the upper brainstem (US) and lower brainstem (LS) exhibited an endogenous circadian one. The peak of the striatal rhythm was noted during the nocturnal phase, while that of the brainstem rhythm occurred at 2400 or 0600 hours, regardless of the lighting cycle used.

3. Serotonin levels of rat brain exhibited differential circadian patterns depending upon the region examined. Striatal and upper brainstem 5-HT content varied in an exogenous circadian pattern with a diurnal peak. The rhythm

of 5-HT levels of the LS exhibited a circadian pattern with a nocturnal peak on the normal light cycle. Reversal of the light cycle appeared to cause a flattening of the rhythm, possibly because of the presence in this tissue of concomitant endogenous and exogenous rhythms.

4. The GABA levels of the rat frontal cerebral lobe, cerebellum, and corpora quadrigemina fluctuated in an exogenous circadian pattern with a nocturnal maxima. The GABA rhythm of the TH-HT appeared to represent the interaction of two or more patterns, since an exogenous circadian pattern with marked fluctuation was noted in this area.

5. Circadian patterns were found in glycine levels in most regions of the CNS examined. Patterns with sharp nocturnal peaks were noted in the pons and thoracic cord, while rhythms with sustained maximal levels throughout the dark phase were found in the cervical and lumbar enlargements. No definitive pattern was noted in the medulla. The rhythms of the spinal cord enlargements were thought to be related to the inhibitory functions of glycine in interneuronal inhibition.

6. Photoperiod reversal appeared to cause some alterations in catecholamine metabolism, while it had little effect

on the levels of 5-HT or GABA. Dopamine content of the CS and LS is significantly elevated by the reversed light cycle. Norepinephrine levels of the CS and LS also exhibited a significantly increased peak level over that seen on the normal cycle, while the basal level of NE in the US was elevated by photoperiod reversal. These effects on regional catecholamine content are indicative of the adaptation stress due to photoperiod reversal.

7. An examination of the susceptibility of vehicle-treated mice to MES seizure in different phases of the programmed lighting schedule revealed only one significant alteration in seizure component timing. The extensor latency time was shorter in duration during the dark phase. No change was noted in the minimal electroshock seizure threshold. A comparison of the two control groups, saline-treated versus acacia-treated, revealed a significantly increased tonic extension time in the acacia-treated group.

8. Peak toxicity of the convulsant compounds allylglycine and strychnine in mice occurred during the light phase. This peak toxicity corresponded with the phase in which minimal GABA and glycine levels occur in rodent species. The data suggest that information regarding the action of such agents on biochemical systems can be obtained in

studies of 24-hour fluctuation in drug effect.

9. The anti-convulsant action of acetazolamide, diphenylhydantoin, and phenobarbital in the rat or mouse was unchanged when evaluated in light and dark phases. Diphenylhydantoin and phenobarbital gave similar changes in seizure component times in both rodent species: increased extensor latency during both phases in both species, and increased duration of clonus with decreased tonic extension time in both phases in the mouse and during the light phase in the rat. These phase differences in the rat suggest some finer differences in the metabolism or responsiveness between the two species.

10. The PD50 of meprobamate was significantly elevated during the dark phase in the mouse. A similar elevation of borderline significance occurred in the rat. This fluctuation of the PD50 appeared to be due to an alteration of the animal's responsiveness to the general depressant actions of meprobamate.

11. Amino-oxyacetic acid pretreatment in mice prolonged the tonic extension phase, when administered in doses of greater than 50 mg./Kg. No discernible circadian differences were noted in this response. Although potent anticonvulsant actions have been attributed to this agent when

seizure is induced via skull electrodes, these effects are apparently lacking when MES is administered via corneal electrodes.

CHAPTER SEVEN

APPENDIX

Appendix A

Cortical GABA levels of rats on a "normal" illumination cycle.*

Table 14. GABA levels are expressed in micromoles/gram WTW plus or minus their respective standard deviation (n = 6).

TIME	GABA, mcM./gm. WTW
0600	2.68 ± 0.26
1200	2.33 ± 0.43
1800	2.38 ± 0.45
2400	2.47 ± 0.32

*This study was conducted in the month of March, 1970.

Appendix B

A comparison of the circadian fluctuations of dopamine and homovanillic acid levels in rat CS revealed an inverse relationship (Figure 27 & 28).

HVA levels were determined by the method of Juorio and co-workers (1966). The method involved the separation of HVA from an acetic acid brain extract on a Dowex 1X2 column (0.4 x 2.0-2.5 cm.). The column was washed with distilled water, and HVA was eluted with 0.1N HCl. A portion of the acid eluate was developed for fluorimetric quantification by addition of 5N NH_4OH containing $FeCl_3$, 20 mcg./ml. The reaction was stopped after four minutes by addition of 0.1% cysteine hydrochloride, and fluorescense was read at 320 mm. (excitation) and 425 mm. (emission) on an Aminco-Bowman SPF (slit scheme 2).

The biphasic rhythm was probably produced by the housing of these animals in our general research laboratory. Thus, a peak occurred at the mid-dark phase, with a supplemental peak occurring in the midafternoon, when environmental stimuli and feeding led to arousal in these normally quiescent animals. These circumstances caused an increase in motor activity during the afternoon.

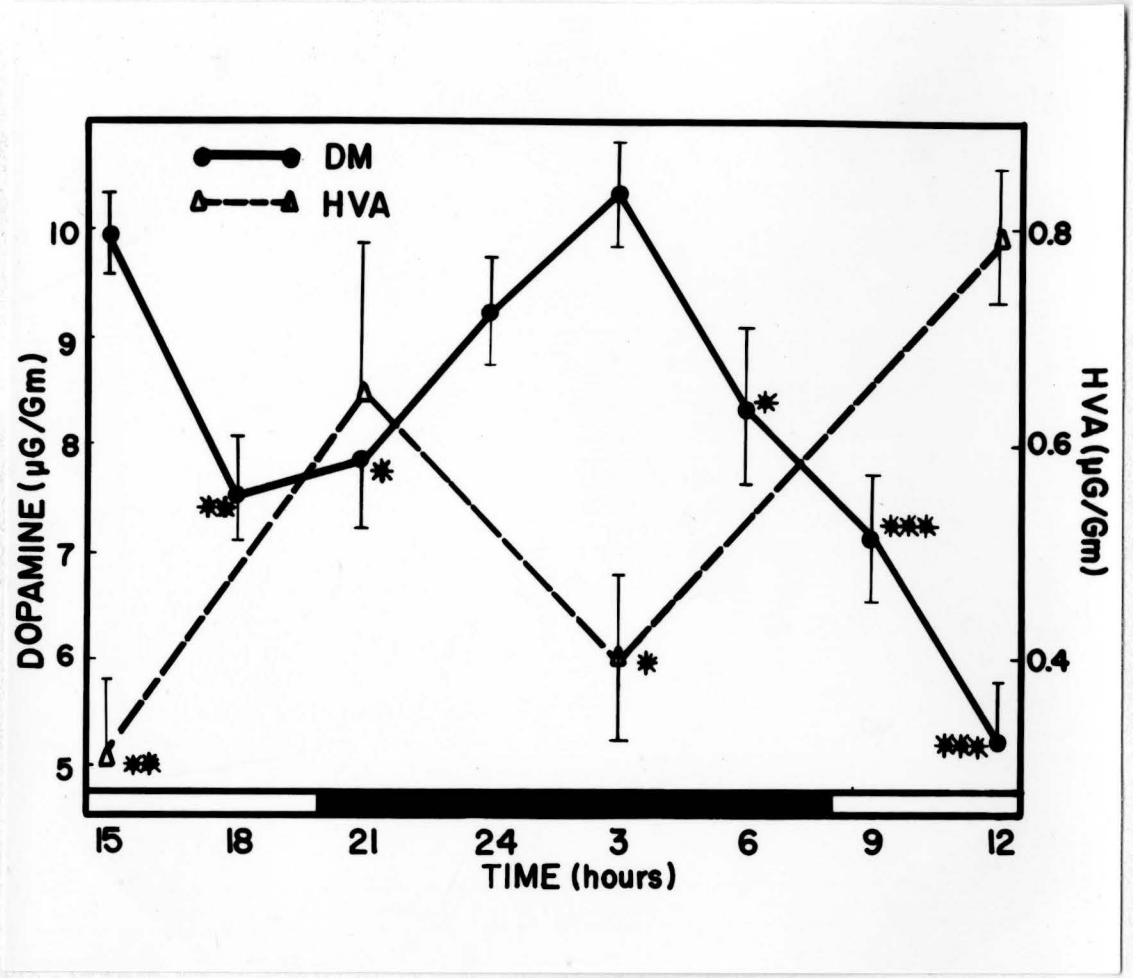


Figure 27. The concentration of DM and HVA in the rat corpus striatum as a function of time. DM content is expressed in mcg./gm. WTW on the left ordinate. The concentration of HVA in mcg./gm. WTW is expressed on the right ordinate. Time is expressed on the abscissa in hours CST. The black bar indicates the dark phase of the illumination cycle. Vertical brackets are used to indicate the standard error of the mean (n = 6).

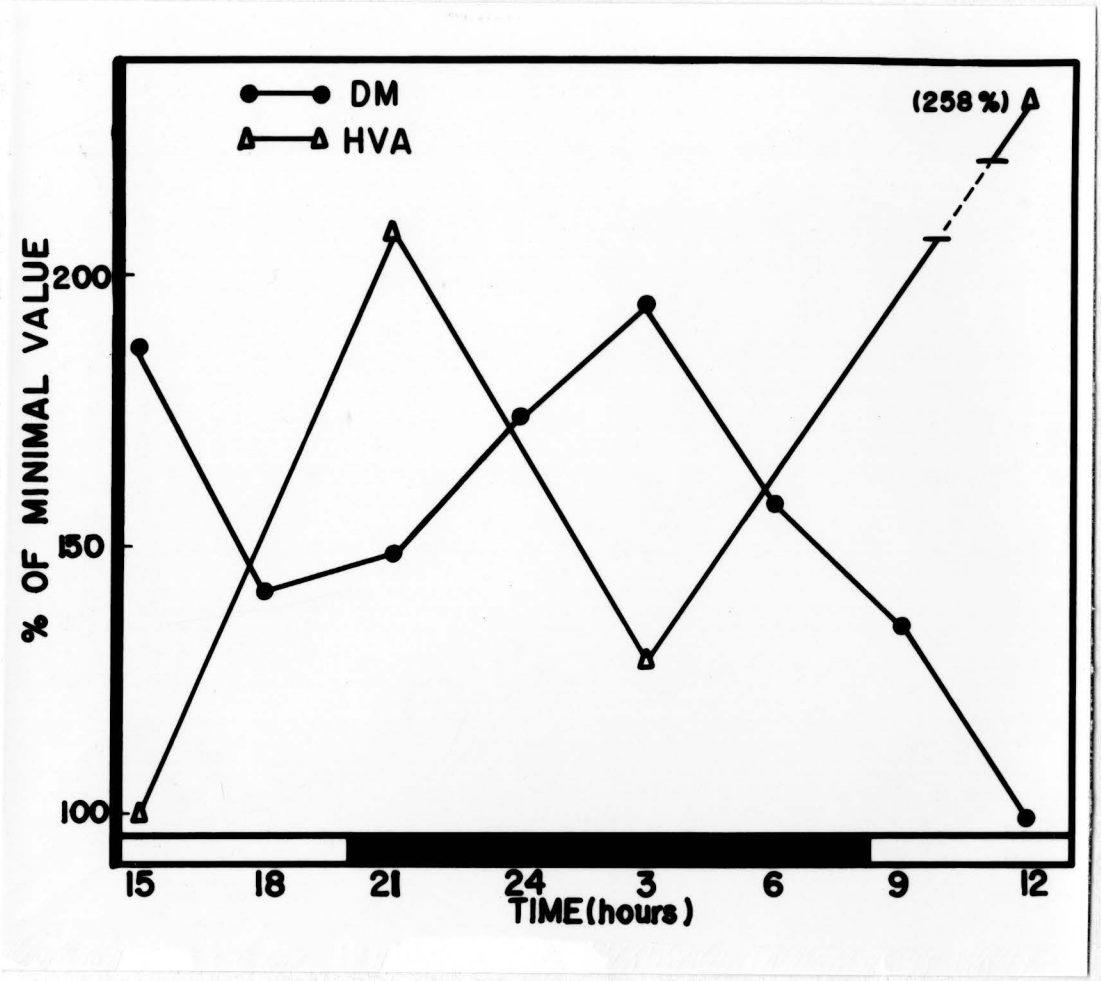


Figure 28. The concentration of DM and HVA in rat corpus striatum as a function of time. The levels of DM and HVA from the previous figure are expressed as a percentage of the minimal, or trough, value. The percentage is indicated on the ordinate. The abscissa is as for the previous figure.

Appendix C

The analysis of variance (ANOV) was also performed on the pooled data to investigate the significance of the differences noted between tissues, phases, and cycles. Interactions between tissues and phases, tissues and cycles, phases and cycles, and tissues and phases and cycles were also examined by this method.

The 3-way ANOV determination was performed at the University of Illinois College of Pharmacy, using a standardized program on the Wang 700-A computer (a description of this technique can be found in "Biostatics in Pharmacological Testing" by C.W. Dunnett in Selected Pharmacological Testing Methods, Volume 3, Marcel Dekker, Inc.: New York, 1968).

The P-values are summarized in Table 15. It is evident from this table that statistically significant differences were noted only between pooled levels of DM and of glycine. Significant differences between the normal and reverse cycles were noted in the case of DM and GABA levels of the CTX and CBLM indicating the possibility of a lunar or annual influence in these rhythms.

A tissue-phase interaction was noted in the case of DM content. This is not surprising as other statistical

COMPARISONS	DM	NE	5-HT	GABA (CQ/ TH-HT)	GABA (CTX/ CBLM)	GLY
Tissue-tissue	<0.01	<0.01	<0.01	<0.01	<0.01	0.05
Light phase-Dark phase	<0.01	>0.05	>0.05	0.05	>0.05	0.05
Normal cycle-Reverse cycle	0.05	>0.05	>0.05	>0.05	<0.01	----
INTERACTIONS						
Tissue-phase	<0.01	>0.05	>0.05	>0.05	>0.05	>0.05
Tissue-cycle	>0.05	>0.05	>0.05	>0.05	>0.05	----
Phase-cycle	>0.05	<0.01	>0.05	>0.05	>0.05	----
Tissue-phase-cycle	>0.05	0.01	>0.05	>0.05	>0.05	----

Table 15. Summary of probability values determined by analysis of variance. Compounds are indicated horizontally and the various items compared are described in the vertical column at the left.

procedures had indicated a reversal in only one of the rhythms tested. When tissue-cycle interactions were tested, no significant differences were found. NE levels exhibited significant differences when tested for both phase-cycle and tissue-phase-cycle interactions.

From this finding it is apparent that reversal in the amine rhythm was not complete for the period of the reversed illumination cycle used. This fact was evident and noted earlier when it was shown that reversal of the brainstem NE did not occur.

This analysis differs from that utilized in the thesis in that only pooled data was considered. The findings, however, are in general agreement with those of the Results Section. Some of the significant differences obtained between peak and trough values by techniques normally used in chronobiology are lost when data is thusly pooled.

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APPROVAL SHEET

The dissertation submitted by Robert W. Piepho has been read and approved by five members of the faculty of Loyola University, Stritch School of Medicine.

The final copies of the dissertation have been examined by the director of the examining committee and the signature which appears below verifies the fact that all necessary changes have been incorporated and that the dissertation is now given final approval with reference to content, form and mechanical accuracy.

The dissertation is, therefore, accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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